39. (new) The isolated nucleic acid of claim 38, wherein the nucleic acid comprises SEQ ID NO:1.

40. (new) The isolated nucleic acid of claim 38, wherein the nucleic acid comprises SEQ ID NO:3.

41. (new) An expression vector comprising the nucleic acid of claim 38.

42. (new) A host cell comprising the nucleic acid of claim 41.

REMARKS

With entry of the current amendment, claims 7, 8, and 31 have been cancelled, new claims 38-42 have been added and claims 1, 6, 19, 24, 30, and 36 have been amended. Accordingly, claims 1-6, 19-24, 26, 30, 32, 33, and 35-42 are currently under examination. A copy of the claims under examination is provided in Appendix B, attached hereto.

The amendment to the specification adds no new matter. Support for the amendment is provided in Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Publish., Cold Spring Harbor, NY, 2nd ed. (1989), which is cited in the specification at page 15 line 30 through page 16, line 31, and is incorporated by reference (page 54, lines 8-10). The amendment to the specification is supported in multiple passages in Sambrook, in particular in Chapter 1, *e.g.*, page 1.101; Chapter 7, *e.g.*, page 7.52; and Chapter 9, pages 9.47-9.55. The two sentences that are added in the amendment are from page 9.47, first sentence of the page, and first sentence of the passage designated "1". A copy of page 9.47 is attached hereto as Appendix C.

The amendments to the claims add no new matter and are supported throughout the application as filed.

For convenience, the rejections are addressed in the order presented in the Office Action mailed September 10, 2002.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 19-23 and 31 were rejected as allegedly indefinite in the recitation of the term "essentially". Although Applicants disagree with the Examiner for reasons of record, in order to expedite prosecution, claim 19 has been amended to recite a nucleotide sequence a nucleotide sequence that encodes a human menin as set forth in SEQ ID NO:2. With respect to claim 31, the rejection is moot in view of the cancellation of the claims. Applicants therefore respectfully request withdrawal of the rejection.

Claim 31 was rejected as allegedly indefinite with regard to stringent hybridization conditions. The rejection is moot in view of the cancellation of the claim. Applicants therefore respectfully request withdrawal of the rejection.

Maintained rejection under 35 U.S.C. § 112, first paragraph--enablement

Claims 1-3, 5, 8, 24, 26, 30-33, and 36-37 stand rejection as allegedly not enabled. The rejection alleges that the claims are not enabled because it would require undue experimentation to identify nucleic acids having at least 60% identity to the reference sequence or that specifically bind polyclonal antibodies to SEQ ID NO:2. In particular, the Examiner maintains that Applicants' arguments filed June 19, 2002 were not persuasive because antibodies bind epitopes and no specific epitopes are identified in the specification. The Examiner reasoned that one in the art cannot identify antibodies that specifically bind to a protein in the absence of the knowledge of the epitope to which it binds. To the extent that the rejection applies to the amended claims, Applicants respectfully traverse.

As noted in Applicants' previous response, the specification teaches the preparation of antibodies that have specificity for a protein or protein family, and moreover, points to teachings in the art, *e.g.*, Harlow and Lane (cited at page 13, lines 7-10 of the specification), that provide additional guidance in developing antibodies,

polyclonal or monoclonal, that specifically bind an antigen. Applicants further submit that binding specificity in reference to antibody binding to a protein, *i.e.,* an antigen, is well known in the art. For example, Harlow & Lane refer to antigen-specific antibodies at page 313, attached as Appendix D. Here, they teach immunoaffinity purification of polyclonal antibodies. They state that "antibodies within the polyclonal pool that are specific for the antigen are allowed to bind. The unbound antibodies are removed by washing, and the specific antibodies are eluted." The passage continues, stating that a "major strength of this method is its unique ability to isolate specific antibodies from a mixed pool." Thus, binding specificity in reference to a protein, not just a particular epitope, is commonly used in the art.

Furthermore, as noted in the previous response, the specification enables one of skill in the art to identify nucleic acids that encoding proteins having at least 60% amino acid sequence identity to SEQ ID NO:2 without undue experimentation. Applicants submit herewith two publications (Guru *et al., Mammalian Genome* 10:592-596, 1999; and Manickam *et al. Mammalian Genome* 11:448-454, 2000, attached hereto as Appendices E and F, respectively) that teach such sequences. The sequences were identified using routine techniques (*e.g.,* sequence comparison, library screening, northern blot analysis) that are well known in the art and described in the specification to provide additional guidance to the practitioner. In particular, Guru *et al.* teach the isolation, genomic organization and expression of a mouse *MEN1* gene. The amino acid sequence encoded by the mouse gene has 97% identity to human menin (*see, e.g.,* Figure 1, page 593), which has the sequence set forth in SEQ ID NO:2. Using a *men1* cDNA probe, the authors show that the gene is expressed using western blotting (page 593, Figure 1b) and Northern analysis (page 595). Similarly, Manickam *et al.* teach the identification of zebrafish cDNA and genomic *Men1* nucleic acid sequences. The protein encoded by zebrafish *Men1* has 67% identity to human menin (*see, e.g.,* page 450, Figure 3). Applicants additionally note that Manickam *et al.* also refers to a previously identified rat menin that also shares 97% identity with the human sequence (page 451,

first column). Northern analysis showed that the zebrafish *Men1* gene is expressed (page 451).

In summary, these publications provide further evidence that the disclosure in the specification enables the practitioner to identify and characterize the claimed nucleic acid sequences. Applicants therefore respectfully request withdrawal of the rejection.

New Grounds of Objection

Applicants amendment filed June 19, 2002 was objected to as allegedly introducing new matter into the specification. Applicants disagree. "An amendment to correct an obvious error does not constitute new matter where one skilled in the art would not only recognize the existence of error in the specification, but also the appropriate correction" (MPEP § 2163.07(II), citing *In re Oda, 443 F.2d 1200, 170 USPQ 268* (CCPA). The Examiner presents no evidence or reasoning as to why one of skill in the art would not readily recognize that "50% formalin with 1 mg heparin" should be corrected to "50% formamide". However, in order to expedite prosecution, Applicants have cancelled the previous amendment and amended the specification to incorporate two sentences taken from Sambrook, as explained above. Pursuant to MPEP § 608.01(p), Applicants declare that the amendatory material, *i.e.*, the two sentences, is part of the same material that is incorporated by reference in the application.

New Grounds of Rejection--35 U.S.C. § 112, first paragraph-written description

Claims 19-24 and 26 were rejected as allegedly lacking adequate written description. Although Applicants disagree, in order to expedite prosecution, the claims have been amended. Applicants therefore respectfully request withdrawal of the rejection.

Claim 8 was rejected to as allegedly lacking adequate written description of hybridization conditions comprising 50% formamide. The rejection is moot in view of the cancellation of the claim.

New Ground of Rejection--35 U.S.C. § 112, first paragraph-enablement

Claims 1-3, 5, 6, 8, 19-24, 26, 30-33, and 36-37 were rejected as allegedly not enabled. The rejection alleges that the specification does not provide enablement for nucleic acid molecules encoding SEQ ID NO:2. In particular, the Examiner does not appear to believe Applicants' assertion that the nucleic acids that encode menin/SEQ ID NO:2 are actually expressed. The Examiner argues that because protein expression may be regulated at the level of translation, one of skill could not reasonably predict that the claimed sequence would ever be expressed *in vivo*. Applicants respectfully traverse.

First, the Examiner's arguments and examples of translational control do not provide proper evidence or reasoning as to why one of skill in the art could not reasonably expect that an RNA encoding menin would be expressed. The examples provided (ferritin, ornithine decarboxylase, and p53) are in fact expressed as proteins, regardless of the teachings in the art that the levels of the protein can be controlled through translational as well as transcriptional mechanisms. Moreover, Applicants' teach that mutations in the claimed sequences play a role in multiple endocrine neoplasia type 1. The rejection provides no reasoning or evidence as to why one of skill would not expect a gene that plays a role in a disease to be expressed. Accordingly, the rejection has not established that the claims are not enabled.

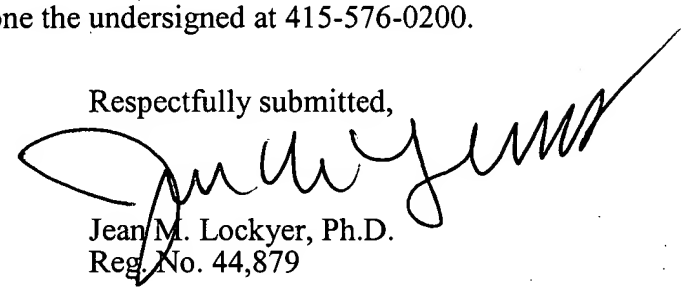
Lastly, in order to expedite prosecution, a paper (Wautot *et al., Int. J. Cancer* 85, 877-881, 2000, attached as Appendix G) submitted herewith provides further evidence showing that menin is expressed. Wautot *et al.* describe the analysis of various cell lines and tissues using polyclonal antibodies to human menin. The results show that menin was expressed in all the cell lines tested and that normal human tissues express menin (page 879, first column and Figure 2). Moreover, the results presented in this publication (*see, e.g.,* the non-human cell lines in Figure 2) and in Guru *et al., supra,* (Figure 1b, page 593) show that related menin proteins, *e.g.,* mouse proteins, are also expressed. Thus, these data present additional evidence that the claims are enabled. Applicants therefore respectfully request withdrawal of the rejection.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

The phrases "hybridizing specifically to" or "hybridizing selectively to" or "selectively or specifically hybridizes", refers to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.,* total cellular) DNA or RNA. "Stringent hybridization" or "stringent conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments, *e.g.*, Southern and Northern hybridizations, are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters of nylon membranes. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% [formalin] formamide [with 1 mg of heparin] at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is: 0.15M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see,*

Sambrook, *supra* for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4 to 6x SSC at 40°C for 15 minutes. As used herein, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids which do not hybridize to each other under stringent conditions can still be substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

1. (twice amended) An isolated or recombinant nucleic acid encoding menin, wherein said nucleic acid encodes a protein defined as follows:

(i) having a calculated molecular weight of about 67.5 kDa; and

(ii) [(a) specifically binding to a specific polyclonal antibody raised against a protein with a sequence as set forth in SEQ ID NO:2; or]

(b)] having at least 60% amino acid sequence identity to a protein with a sequence as set forth in SEQ ID NO:2;

wherein the isolated nucleic acid sequence specifically hybridizes to SEQ ID NO:1 under hybridization conditions comprising 50% formamide at 42°C and stringent wash conditions comprising 0.2XSSC at 65°C for 15 minutes.

6. (amended) The isolated or recombinant nucleic acid of claim [5] 1, wherein the nucleic acid sequence encodes a menin protein that binds to an antibody raised against a polypeptide having an amino acid sequence [with a sequence] as set forth in SEQ ID NO:2.

19. (twice amended) A method for detecting in a test sample the presence or absence of a mutation in a human MEN1 gene comprising a nucleotide sequence [essentially] that encodes [encoding] a human menin as set forth in SEQ ID NO:2, or the presence or absence of a MEN1 allele, the method comprising:

a) contacting said test sample suspected of missing a MEN1 allele or encoding a mutant form of the human menin with a first oligonucleotide having a sequence that discriminates between the wild type gene and the missing allele or mutant form[, wherein the first oligonucleotide specifically hybridizes to a nucleic acid sequence comprising at least 95% identity to SEQ ID NO:3]; and,

b) detecting the formation of a duplex between the gene and the first oligonucleotide sequence.

24. (twice amended) A kit for detecting in a test sample the presence or absence of a mutation in a MEN1 gene comprising a nucleotide sequence encoding a menin polypeptide as set forth in SEQ ID NO:2, the kit comprising;

a) a container holding a first oligonucleotide sequence that discriminates between the wild type gene and the mutant form[, and that specifically hybridizes to a nucleic acid sequence comprising at least 95% identity to SEQ ID NO:3]; and

b) a container holding a reagent for detecting the formation of a duplex between the gene and the first nucleotide sequence.

30. (amended) A transfected cell comprising a heterologous nucleic acid of claim 1 [encoding a menin protein or subsequence thereof].

36. (twice amended) An expression cassette comprising a nucleic acid of claim 1 [encoding a menin polypeptide], wherein the nucleic acid is operably linked to a promoter.

38. (new) An isolated nucleic acid encoding a polypeptide comprising the sequence set forth in SEQ ID NO:2.

39. (new) The isolated nucleic acid of claim 38, wherein the nucleic acid comprises SEQ ID NO:1.

40. (new) The isolated nucleic acid of claim 38, wherein the nucleic acid comprises SEQ ID NO:3.

41. (new) An expression vector comprising the nucleic acid of claim 38.

42. (new) A host cell comprising the nucleic acid of claim 41.

APPENDIX B

CLAIMS CURRENTLY UNDER EXAMINATION

1. (twice amended) An isolated or recombinant nucleic acid encoding menin, wherein said nucleic acid encodes a protein defined as follows:

(i) having a calculated molecular weight of about 67.5 kDa; and

(ii) having at least 60% amino acid sequence identity to a protein with a sequence as set forth in SEQ ID NO:2;

wherein the isolated nucleic acid sequence specifically hybridizes to SEQ ID NO:1 under hybridization conditions comprising 50% formamide at 42°C and stringent wash conditions comprising 0.2XSSC at 65°C for 15 minutes.

2. (as filed) The isolated or recombinant nucleic acid of claim 1, which further comprises non-coding sequence.

3. (as filed) The isolated or recombinant nucleic acid of claim 2, wherein the non-coding sequence comprises introns.

4. (as filed) The isolated or recombinant nucleic acid of claim 3, wherein the sequence is SEQ ID NO:3.

5. (as filed) The isolated or recombinant nucleic acid of claim 1, wherein the nucleic acid sequence encodes a protein having at least 80% amino acid sequence identity to a protein with a sequence as set forth in SEQ ID NO:2.

6. (amended) The isolated or recombinant nucleic acid of claim 1, wherein the nucleic acid sequence encodes a menin protein that binds to an antibody raised against a polypeptide having an amino acid sequence as set forth in SEQ ID NO:2.

19. (twice amended) A method for detecting in a test sample the presence or absence of a mutation in a human MEN1 gene comprising a nucleotide sequence that encodes a human menin as set forth in SEQ ID NO:2, or the presence or absence of a MEN1 allele, the method comprising:

a) contacting said test sample suspected of missing a MEN1 allele or encoding a mutant form of the human menin with a first oligonucleotide having a sequence that discriminates between the wild type gene and the missing allele or mutant form; and,

b) detecting the formation of a duplex between the gene and the first oligonucleotide sequence.

20. (amended) A method of claim 19, wherein the first oligonucleotide is unable to bind to the wild-type MEN1 gene under hybridization conditions in which the first oligonucleotide binds to the mutant sequence of MEN1.

21. (as filed) A method of claim 19, wherein the contacting step further comprises amplifying a portion of the human MEN1 gene and where the first nucleic acid is a polymerase chain reaction amplification primer which binds to an intron of MEN1.

22. (as filed) A method of claim 19, wherein the contacting step further comprises amplifying a portion of MEN1 and where the first nucleic acid is a polymerase chain reaction amplification primer which discriminates between wild-type and mutant forms of MEN1 using allelic specific polymerase chain reaction.

23. (as filed) A method of claim 19, wherein the first nucleic acid binds to either exons or introns of the genomic DNA encoding the human menin gene.

24. (twice amended) A kit for detecting in a test sample the presence or absence of a mutation in a MEN1 gene comprising a nucleotide sequence encoding a menin polypeptide as set forth in SEQ ID NO:2, the kit comprising;

a) a container holding a first oligonucleotide sequence that discriminates between the wild type gene and the mutant form; and

b) a container holding a reagent for detecting the formation of a duplex between the gene and the first nucleotide sequence.

26. (amended) The kit of claim 24, further comprising amplification primer pairs specifically binding to a human genomic DNA sequence encoding menin.

30. (amended) A transfected cell comprising a heterologous nucleic acid of claim 1.

32. (amended) The transfected cell of claim 30, wherein the heterologous or exogenous nucleic acid comprises a nucleic acid as set forth in SEQ ID NO:1 or SEQ ID NO:3.

33. (amended) The transfected cell of claim 30, wherein the cell is a human cell.

36. (twice amended) An expression cassette comprising a nucleic acid of claim 1, wherein the nucleic acid is operably linked to a promoter.

37. (as filed) The expression cassette of claim 36, further comprising an expression vector.

38. (new) An isolated nucleic acid encoding a polypeptide comprising the sequence set forth in SEQ ID NO:2.

39. (new) The isolated nucleic acid of claim 38, wherein the nucleic acid comprises SEQ ID NO:1.

40. (new) The isolated nucleic acid of claim 38, wherein the nucleic acid comprises SEQ ID NO:3.

41. (new) An expression vector comprising the nucleic acid of claim 38.

42. (new) A host cell comprising the nucleic acid of claim 41.

2

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

E.F. Fritsch
GENETICS INSTITUTE

T. Maniatis
HARVARD UNIVERSITY

Cold Spring Harbor Laboratory Press
1989

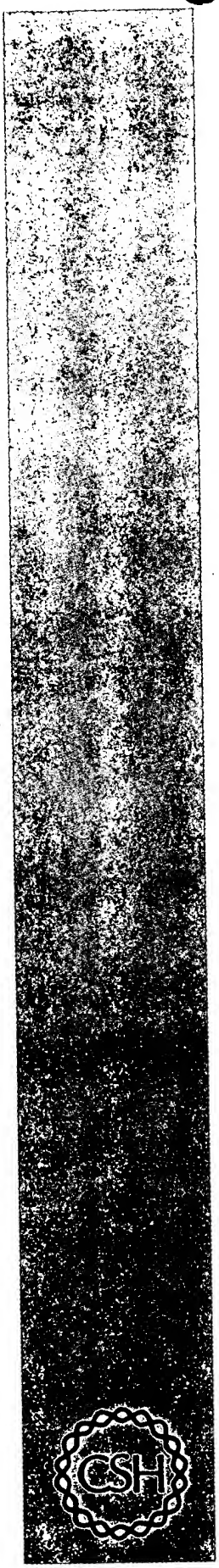
HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the



Antibodies

A LABORATORY MANUAL

Ed Harlow
Cold Spring Harbor Laboratory

David Lane
Imperial Cancer Research Fund Laboratories

Cold Spring Harbor Laboratory
1988

IMMUNOAFFINITY PURIFICATION OF ANTIBODIES ON AN ANTIGEN COLUMN*

The only method commonly used to purify antigen-specific antibodies from a preparation of polyclonal antibodies is immunoaffinity purification. In this procedure pure antigen is bound covalently to a solid support. The antibodies within the polyclonal pool that are specific for the antigen are allowed to bind. The unbound antibodies are removed by washing, and the specific antibodies are eluted. This method is unnecessary for monoclonal antibodies, which are homogeneous in their antigen binding activity.

The major strength of this method is its unique ability to isolate specific antibodies from a mixed pool. Its principal disadvantages are that it requires large amounts of antigen and that elution of the specific antibody requires conditions that can lead to some loss of activity.

For protein and peptide antigens, a large number of procedures have been used to prepare affinity columns capable of isolating specific antibodies. The use of activated beads is recommended for most protein antigens. The various types of activated beads are discussed in detail in Chapter 13. The most commonly used activated bead is cyanogen bromide-activated agarose, although this matrix may not be the best choice for many applications (see p. 532). Coupling to beads can also be used for peptide antigens, preferably by first coupling the peptide to a protein carrier (p. 78). For elution from peptide columns, the protein carrier must be distinct from that used for immunization (e.g., BSA peptides for purification of antibodies raised against KLH peptides). The protein carrier–peptide conjugate is then coupled to the activated beads.

*Adapted from Campbell et al. (1951).

Mammalian Genome 10, 592–596 (1999).

Mammalian Genome
Incorporating Mouse Genome

Isolation, genomic organization, and expression analysis of *Men1*, the murine homolog of the MEN1 gene

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Received: 17 December 1998 / Accepted: 2 February 1999

Abstract. The mouse homolog of the human MEN1 gene, which is defective in a dominant familial cancer syndrome, multiple endocrine neoplasia type 1 (MEN1), has been identified and characterized. The mouse *Men1* transcript contains an open reading frame encoding a protein of 611 amino acids which has 97% identity and 98% similarity to human menin. Sequence of the entire *Men1* gene (9.3 kb) was assembled, revealing 10 exons, with exon 1 being non-coding; a polymorphic tetranucleotide repeat was located in the 5′- flanking region. The exon-intron organization and the size of the coding exons 2–9 were well conserved between the human and mouse genes. Fluorescence in situ hybridization localized the *Men1* gene to mouse Chromosome (Chr) 19, a region known to be syntenic to human Chr 11q13, the locus for the MEN1 gene. Northern analysis indicated two messages—2.7 kb and 3.1 kb—expressed in all stages of the embryo analyzed and in all eight adult tissues tested. The larger transcript differs from the smaller by the inclusion of an unspliced intron 1. Whole-mount in situ hybridization of 10.5-day and 11.5-day embryos showed ubiquitous expression of *Men1* RNA. Western analysis with antibodies raised against a conserved C-terminal peptide identified an approximately 67-kDa protein in the lysates of adult mouse brain, kidney, liver, pancreas, and spleen tissues, consistent with the size of human menin. The levels of mouse menin do not appear to fluctuate during the cell cycle.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant familial cancer syndrome characterized by tumors of the parathyroids, enteropancreatic endocrine tissues, and the anterior pituitary (Metz et al. 1994). The MEN1 locus was mapped to human Chr 11q13 by linkage analysis (Larsson et al. 1988), and the responsible MEN1 gene was identified by positional cloning (Chandrasekharappa et al. 1997). The human MEN1 gene is organized into 10 exons (the first being untranslated) and is ubiquitously expressed as a 2.8-kb transcript. This transcript encodes a 610-amino acid product, termed menin, which exhibits no apparent similarities to any known proteins. Over 200 independent germline and somatic mutations spread throughout the coding region of menin have been described (Marx et al. 1999). Somatic mutations are observed to a variable extent in certain sporadic tumors: parathyroid adenoma, gastrinoma, insulinoma, and lung carcinoid (Marx et al. 1999). MEN1 appears to be a typical tumor suppressor gene: lack of menin owing to inactivation of both alleles probably leads to the development of tumors.

Although no murine syndrome similar to MEN1 has been reported to date, here we detail the identification of the mouse MEN1 homologous gene, *Men1*, on mouse Chr 19. The cDNA sequence, genomic organization, RNA and protein expression patterns are presented and discussed.

Materials and methods

Screening BAC library. The entire Men1 cDNA sequence was assembled by sequencing the insert in a cDNA clone (IMAGE clone ID 557658). DNA pools from a BAC library prepared from 129Sv/cJ7 mouse DNA (Research Genetics, Huntsville, AL) were screened by PCR with primers (5′-GCTGAAGGCGCCCAGAAGACG-3′ and 5′-CTGAGCGGTGAATCGGGCATAGAG-3′) designed from the mouse *Men1* cDNA sequence. Three BAC clones, 331J21, 331K21, and 7D23, were isolated. The size of the inserts was determined by pulsed-field gel electrophoresis of *Not*I-digested BAC DNAs as described earlier (Guru et al. 1997b).

Subcloning and sequence analysis. DNA isolated from the BAC clone 7D23 was subjected to partial digestion with *Sau*3A1 to generate fragments of approx. 10–12 kb. The fragments were cloned into the *Bam*H1 site of the plasmid pBluescriptII KS+ (pBSIIKS+). Colonies were analyzed by PCR with STSs designed for both the 5′- and 3′-ends of the *Men1* cDNA in order to identify the clones containing the entire gene. One clone with an approximate 10-kb insert containing the entire *Men1* gene was chosen for subsequent sequence analysis and genomic characterization.

Primers, UP1 (GACATCCATGGCTACACAGAAAAACCC) and LP1 (GCCTGTGTAAAGGGAAGAAGACAGAGAGAGT), generating a 260-bp product, were used for the PCR amplification of the $(AAAG)_{10}$ repeat alleles from mouse genomic DNA.

Northern analysis. A 900-bp insert representing the 3′-end of the *Men1* cDNA clone (IMAGE clone ID. 402210) was released by digestion of the plasmid DNA with *Eco*RI and *Not*I, labeled with a random primer labeling kit (Amersham) and hybridized to a mouse multiple tissue Northern blot (#7762-1) and embryo blot (#7763-1) (Clontech, Palo Alto, CA) as described earlier (Guru et al. 1997a). For the 5′-end probe, a 299-bp PCR product representing most of intron 1 was amplified from the BAC clone 331J21.

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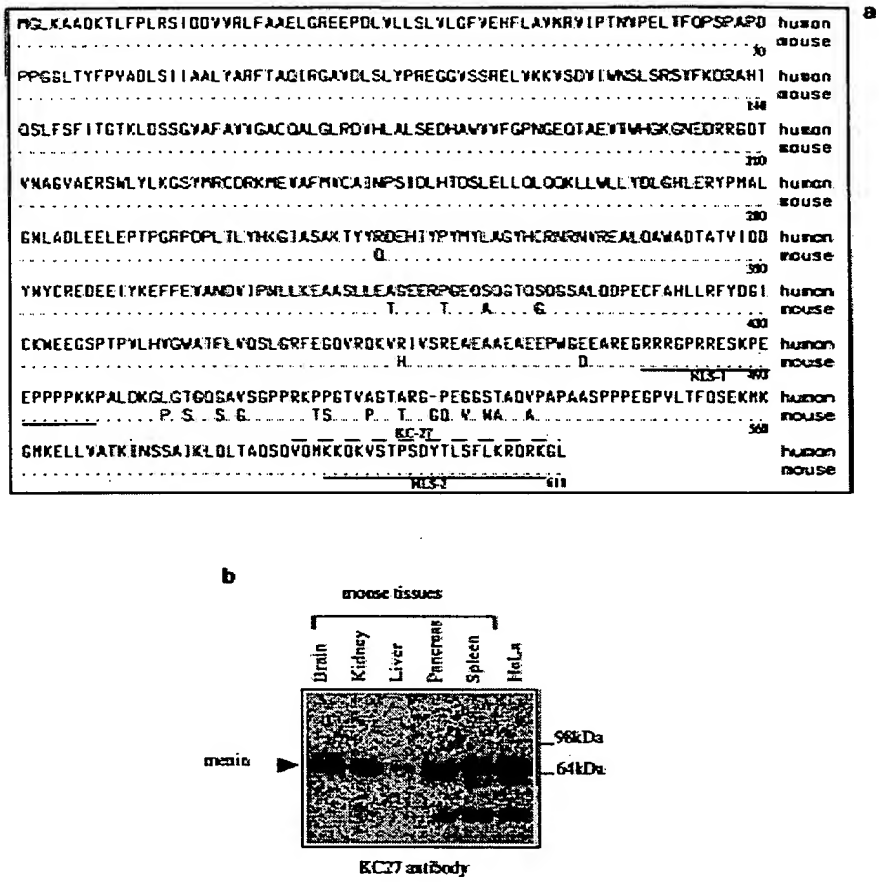

Fig. 1. a) Comparison of human and mouse menin sequences. Alignment of the amino acid sequence was carried out using Multiple Sequence Alignment program of DNASTAR. The human and mouse menin sequences are shown on the top and bottom respectively. An identical amino acid in mouse menin is indicated by a dot. The numbering of amino acid refers to the mouse protein sequence. The C-terminal 27 aa sequence used for producing a rabbit antibody (KC-27) is indicated with a broken line on top of the sequence. The two nuclear localization signals (NLS-1 and NLS-2) mapped in human menin are underlined. b) Western analysis of protein lysates from five adult mouse tissues and HeLa cells (human) probed with KC27 antibodies. The location of menin is indicated on the left, and those of size markers on the right.

Western analysis. Mouse tissues were homogenized in Tris-HCl buffer [50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.3 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 0.5% NP-40, 100 μg/ml AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], 1 μg/ml aprotinin/leupeptin] and centrifuged at 20,000 *g* for 1 h. The supernatant was mixed with Laemmli buffer, boiled for 5 min, and then the proteins (40 μg for each tissue and 10 μg for HeLa cell lysate) were separated on a 10–20% SDS gel. Production of KC27 antibodies in rabbits to a C-terminal human menin 27 aa peptide and routine Western analysis procedures were as described previously (Guru et al. 1998).

Fluorescence in situ hybridization (FISH). Preparation of metaphase chromosomes from lymphocytes obtained from mouse spleen, hybridization conditions, and analysis of fluorescent signals were as described previously (Pecker et al. 1996). The BAC clone (331J21) containing the mouse *Men1* gene was labeled by nick translation with biotin-conjugated dUTP. In order to facilitate chromosome identification, a digoxigenin-labeled mouse Chr 19-specific painting probe was used for co-hybridization. The biotinylated probes were detected by incubation with avidin-FITC, and the digoxigenin sequences were detected with mouse anti-digoxin and goat anti-mouse conjugated to TRITC (Sigma Chemical Co., St. Louis, Mo.). Chromosomes were counterstained with DAPI.

Whole-mount mouse embryo in situ hybridization. Non-radioactive in situ hybridization was performed on whole embryos [10.5 and 11.5 days post coitum (p.c.)] and cryosections (12.5 days p.c.) prepared as previously described (Kos et al. 1999). Embryos were obtained from matings of FVB mice. Noon of the plug day was considered 0.5 days p.c., but embryonic ages were confirmed by comparison of somite number and external features with descriptions by Kaufman (1992). Sense and antisense digoxigenin-labeled riboprobes were generated from linearized templates by in vitro transcription with T3 or T7 RNA polymerase. A plasmid (IMAGE clone ID. 402210) with an insert representing the 900-bp 3'-end of the *Men1* cDNA was used as a template. For antisense probe, the plasmid was linearized with *Eco*RI and transcribed with T3 RNA polymerase. For sense probe (negative control), the plasmid was linearized with *Not*I and transcribed with T7 RNA polymerase.

Cell synchronization. Synchronization of cells, preparation of protein lysates, and Western analysis were as described earlier (Brown et al. 1997). Briefly, NIH-3T3 cells were synchronized in G_0 by culturing the cells for 96 h under reduced serum [0.5% fetal calf serum (FCS)] conditions in Dulbecco's Modified Eagle Medium (DMEM). To collect cells synchronized in G_1, serum-starved cells were replated in complete growth medium (DMEM supplemented with 10% FCS) for 6 h. Mitotic (M) cells were obtained by culturing in complete growth medium supplemented with colcemid (0.1 μg/ml) for 24 h, and mitotic cells were collected by mitotic shake-off. Extracts of NIH-3T3 cells in log phase growth (Log) were obtained from asynchronous cultures grown in complete growth medium. Aliquots (1×10^6 cells) of asynchronous and synchronized cell populations were stained with propidium idodide and analyzed by flow cytometry to confirm their stage in cell cycle.

Results

Mouse Men1 cDNA sequence and its encoded 611 aa mouse menin protein. A search of the NCBI EST database (dbEST) with human MEN1 cDNA sequence revealed the availability of several mouse cDNA clones with similar sequences. Analysis of the homologous regions in the available EST sequences and the sizes of the clone inserts resulted in identification of the longest cDNA clone (IMAGE clone ID. 557658). A 2984-bp sequence (Genbank acc. no. AF109389) was assembled for the entire cDNA insert in this clone. The sequence includes an ORF encoding the putative mouse menin protein of 611 aa, one amino acid longer than that of human menin (610 aa). The encoded mouse protein shows 97% identity

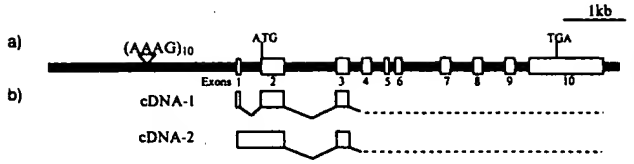

Fig. 2. a) Exon–intron organization of the *Men1* gene. The 9330-bp genomic sequence is shown as a thick horizontal line, and the locations of the 10 exons (open boxes), the tetranucleotide repeat $(AAAG)_{10}$ in the 5′-flanking region, the initiation codon ATG in exon 2, and the termination codon (TGA) in exon 10 are shown. The exon numbers are indicated below. b) A diagram depicting the differences near the 5′-end of the two types of transcripts: cDNA-1, completely spliced 2592-bp transcript. cDNA-2, a 2984-bp transcript with unspliced intron 1. The dotted line indicates that there are no changes in the remaining part of the two transcripts.

with and 98% similarity to human menin sequence (Fig. 1a). Antibodies (KC27) raised against a conserved C-terminal peptide (indicated in Fig. 1a) were used for the identification of mouse menin by Western analysis of lysates from adult mouse brain, kidney, liver, pancreas, and spleen tissues. All the tissues showed a protein of approx. 67 kDa, similar in size to that of human menin (Guru et al. 1998) from HeLa cell lysate (Fig. 1b).

Men1 gene sequence and exon-intron organization. Three BAC clones were isolated by screening a mouse BAC library with an STS specific for the Men1 cDNA sequence. The inserts in clones 331J21 and 331K21 were similar in size (280 kb), whereas that of the clone 7D23 was 120 kb. In addition to their similar size, the identical *Not*I enzyme restriction pattern and their adjacency in the library suggested that the clones 331J21 and 331K21 are likely to be copies. PCR analysis with primers amplifying both ends of the mouse Men1 cDNA sequence revealed that all BAC clones had the entire *Men1* gene.

In order to obtain genomic sequence of the *Men1* gene, a plasmid library was prepared by cloning *Sau*3A1 digests of the BAC clone 7D23 into the *Bam*H1 site of pBSIIKS+. PCR analysis of plasmid clones identified a clone with an approximate 10-kb insert containing the entire *Men1* gene, and a 9286-bp sequence (Genbank acc. no. AF109390) of the mouse gene was assembled by sequencing this insert. The genomic sequence revealed that, similar to the human gene, the mouse gene also consists of 10 exons, and the locations of the initiation (ATG) and the termination codons are in exons 2 and 10, respectively (Fig. 2a). The sizes of the intervening exons 2–9 are identical in human and mouse. Comparison of homologous mouse ESTs in the dbEST database to the assembled Men1 genomic and full-length cDNA sequences indicated two types of cDNA clones. One resembled the human, and transcripts with an unspliced intron 1 (362 bp) along with the otherwise fully processed transcripts contributed the second type of mouse Men1 cDNA clones (Fig. 2b). However, the 611-aa ORF with the translation initiation point (ATG) in exon 2 remained unaltered in both types of messages. A single variant polyadenylation signal (GATAAA) is located 20 nucleotides upstream of the poly-A stretch in all seven Men1 cDNA clones analyzed.

A tetranucleotide repeat $(AAAG)_{10}$ was observed about 1.5 kb upstream of the 1st exon. PCR amplification of the repeat region in DNA samples from various strains of mice revealed four different alleles indicating that this repeat is polymorphic. Allele sizes in *DBA/2J, FVB/NJ,* and *M. spretus* are different from each other. The $(AAAG)_{10}$ allele was observed in C57bl/6J, A/J, and 129/SvJ mice (data not shown).

Chromosomal localization of the Men1 gene by FISH. Chromosomal localization of the *Men1* gene was carried out by FISH analysis of mouse metaphase chromosomes. A biotin-labeled

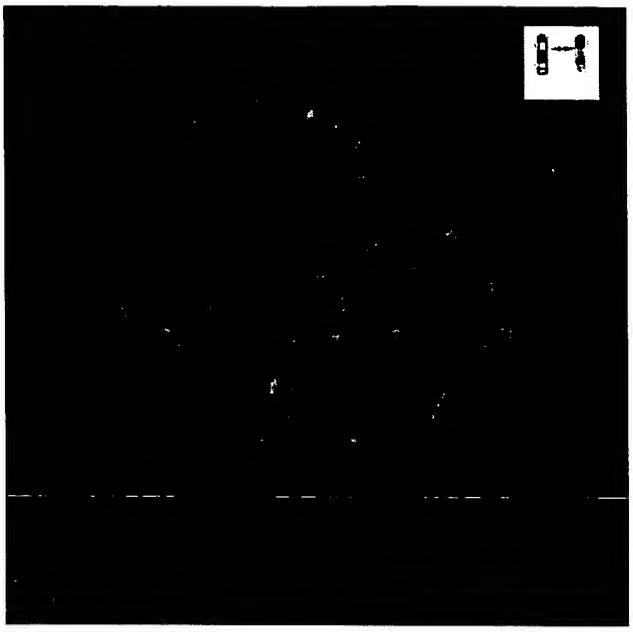

Fig. 3. Fluorescent in situ hybridization of *Men1* gene to mouse metaphase chromosomes. Labeled BAC clone 331J21 DNA (green signal) and Chr 19-specific painting probes (red signal) were cohybridized to mouse metaphase chromosomes. The location of the green signal owing to hybridization of *Men1* containing BAC DNA on the proximal region of Chr 19, between cytogenetic bands B and C2, is depicted in the inset.

331J21 BAC DNA probe was cohybridized with a digoxigenin-labeled mouse Chr 19-specific painting probe. The BAC clone (green signal) hybridized to the proximal region of mouse Chr 19 (red signal; Fig. 3). This region Chr 19 is syntenic with human pericentric Chr 11q13 containing the MEN1 gene. The BAC clone hybridization signals were observed exclusively at Chr 19.

Men1 gene expression by Northern analysis. A multiple-tissue Northern blot representing RNA from eight adult mouse tissues (Clontech) was probed with a labeled 900-bp cDNA insert representing the 3′-end of the Men1 cDNA. The presence of two messages, sized at 2.7 kb and 3.1 kb, was observed in all tissues, although the extent of expression between the tissues varied considerably (Fig. 4a). The expressions in heart, spleen, and skeletal muscle were reduced compared with brain, lung, liver, kidney, and testis. Both messages appear to be expressed in nearly equal amounts in all tissues except for brain, where the longer transcript was prominent. In order to resolve the nature of the two messages, a 299-bp probe generated by PCR amplification of most of intron 1 was hybridized to the same Northern blot. Only the longer 3.1-kb message hybridized to the intron 1 probe, indicating that the longer message originated owing to an unspliced intron 1 (Fig. 4b).

Analysis of *Men1* gene expression during embryonic development with a Northern blot with RNAs from 7-, 11-, 15-, and 17-day embryos showed that both messages (2.7 kb and 3.1 kb) were expressed at all these stages (Fig. 4c). Minimal variation was observed as to the relative amounts of the two messages.

Whole-mount in situ hybridization. In situ hybridization was used to examine the distribution of Men1 mRNA during mouse embryogenesis. Wild-type embryos were examined by whole-mount in situ hybridization at 10.5 and 11.5 days p.c. and by hybridization to cryosections at 12.5 days p.c. At all ages examined, Men1 mRNA appeared to be ubiquitously expressed throghout the embryos. Hybridization result from the 11.5 day p.c. embryo is shown in Fig. 5. Examination of cross sections indicated a higher level of

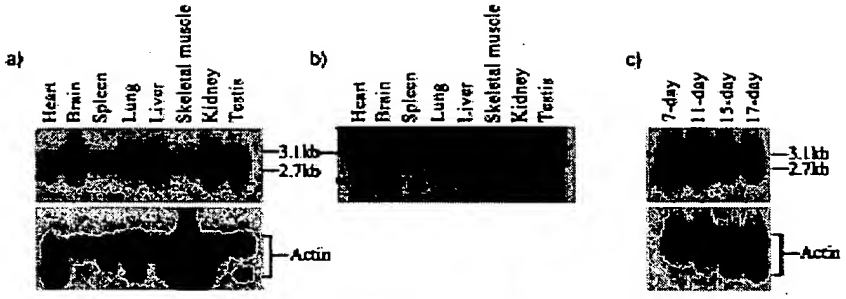

Fig. 4. *Men1*, gene expression by Northern blot analysis. a) A multiple-tissue Northern blot (Clontech) with RNA from eight adult mouse tissues (indicated on the top) was hybridized to a 900-bp radiolabeled probe representing the 3′-end of the *Men1* cDNA. Size and location of the longer (3.1 kb) and the smaller (2.7 kb) messages are indicated. The signals obtained from a beta-actin probe used as a control for RNA loading on this blot are shown at the bottom. b) The same blot was probed with a radiolabeled 299-bp probe representing intron 1. Hybridization of this probe only to the longer 3.1 kb message is indicated. c) Hybridization of a Northern blot containing RNA from 7-, 11-, 15- and 17-day mouse embryos with the 900-bp Men1 cDNA probe as in (a). The locations of the 3.1-kb and 2.7-kb messages are shown. Hybridization of this blot to a control beta-actin probe is shown below.

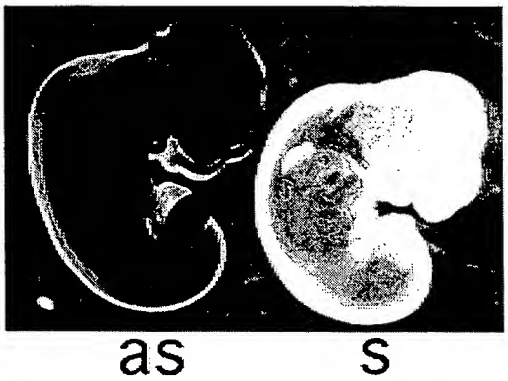

Fig. 5. Expression of *Men1* in mouse embryos by whole-mount in situ hybridization. Antisense (as) and sense (s; control) RNA probes generated by T7 and T3 RNA polymerase with a cDNA clone representing the 900 bp from the 3′-end of Men1 cDNA were hybridized to an 11.5-day embryo.

expression in cranial ganglia, sensory ganglia, and neural tube than observed in adjacent tissues.

Mouse menin expression in the cell cycle. Cultures of the murine cell line NIH-3T3 were synchronized at various points in the cell cycle (G_0, G_1, and M phase) and, along with asynchronous cultures of cells in log-phase growth (Log), were analyzed by Western analysis with KC27 antibodies. The menin levels in all these lysates were equivalent, indicating that menin expression does not fluctuate during the cell cycle.

Discussion

The mouse homolog (*Men1*) of the human MEN1 gene, located as expected at the syntenic region on Chr 19, has been isolated and characterized. A similar exon-intron structure, identical sizes of coding exons, 88% similarity of the coding nucleotide sequence, and 97% identity of the encoded proteins indicate that the *Men1* gene is highly conserved through evolution. In addition to an extra amino acid in the mouse menin, there are only 20 locations where the amino acids differ in the entire length of the two proteins (Fig. 1a). It is interesting to note that none of the 46 amino acids involved in disease-associated germline and/or somatic missense mutations or inframe deletions are different in mouse menin, showing the importance of conservation in defining the structural and functional role of menin. The two nuclear localization signals (Guru et al. 1998) have been completely conserved in mouse (Fig. 1a). Therefore, mouse menin is also expected to be predominantly located in the nucleus.

The sequence of the entire mouse *Men1* gene (9.3 kb) is also reported here (AF109390). A polymorphic tetranucleotide repeat present in the 5′-flank of the *Men1* gene may be a helpful genetic marker in looking for loss of heterozygosity in mouse tumors in which this gene might play a role. While this manuscript was in preparation and revision, two other groups independently reported an analysis of the mouse *Men1* gene (Stewart et al. 1998; Bassett et al. 1999). Our results are in agreement with these two reports. The overlapping sequences of Men1 reported here (Genbank acc. no. AF109389) and those of Stewart and associates (Genbank acc. no. AF016398) are identical, but differ from the sequences reported by Bassett and coworkers (Genbank acc. no. AF072755) at three locations. These differences are: at codons 457 (I changing to M), 466 (E changing to G), and 512 (S changing to L). In these three positions, the sequences reported here are conserved and identical to that of the human menin sequence. The sequences we assembled from seven independent cDNA clones for the 3′ end indicate a GATAAA sequence, located 19 nt upstream of the polyA stretch, as the likely polyadenylation signal.

The mouse *Men1* gene is expressed in at least two alternate forms—an additional isoform arises owing to alternative splicing of intron 1. Both types of messages appear to be expressed nearly equally. The ORF and, therefore, the sequence of the encoded mouse menin are unaltered in these two messages, but alternative translational efficiency is possible.

Northern analysis indicates widespread expression of *Men1* in all embryonic stages (7-day to 17-day) and in all eight adult tissues tested. The significance of the modest expression level differences between different tissues and at different embryonic stages, if any, is unclear.

The distribution of Men1 mRNA was also examined by whole mount in situ hybridization. In mouse embryos, *Men1* was ubiquitously expressed, with somewhat higher levels in neural tissues. The ubiquitous expression and early embryonic expression of mouse menin is suggestive of its fundamental role in a widespread biological function. The molecular basis for the development of neoplasia in restricted endocrine tissues in patients with MEN1, despite ubiquitous expression of the gene, remains to be determined. It is possible that other genes are able to compensate for lost MEN1 function in the unaffected tissues. Analysis of organ function in mice with engineered alterations in *Men1* will be useful for understanding the role of *Men1* in development and disease.

Antibodies raised against a C-terminal peptide that is identical in human and mouse allowed identification of mouse menin by Western analysis (Fig. 1b). The size of the mouse protein, as expected, is similar to that of the human, and levels of menin do not appear to fluctuate during the cell cycle (Fig. 6). These anti-

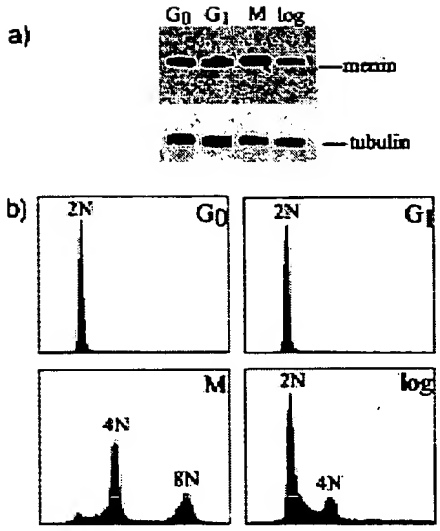

Fig. 6. Mouse menin levels in NIH-3T3 cells at different stages of cell cycle. a) Using the KC27 antibody, menin levels were analyzed by Western blot (whole cell extract; 10 μg protein each lane) from extracts derived from NIH-3T3 cells synchronized in the G_0, G_1, and Mitotic (M) phases of the cell cycle, as well as cells from asynchronous cultures in log phase of the cell growth (Log). The same blot was probed with a tubulin antibody to confirm that equivalent amounts of protein were present in each lane. b) Aliquots of cells were stained with propidium iodide and subjected to flow cytometric analysis. Shown are histograms where cell number is plotted on the Y-axis and DNA content on the X-axis, confirming the cell cycle stage of the synchronized cell populations analyzed in a.

bodies may be useful in future biochemical characterization of mouse menin.

This characterization of the mouse *Men1* gene and its protein product should now set the stage for development of a mouse model of the human disease.

Acknowledgments. We thank Sunita Agarwal for critical reading of the manuscript.

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Isolation, characterization, expression and functional analysis of the zebrafish ortholog of *MEN1*

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Received: 14 January 2000 / Accepted: 18 February 2000

Abstract. Mutations in the *MEN1* gene lead to an autosomal dominant disorder, multiple endocrine neoplasia type 1 (MEN1), which is characterized by tumors of the parathyroid, entero-pancreatic neuroendocrine, and pituitary tissues. The protein encoded by *MEN1*, 610-amino acid menin, resides primarily in the nucleus and binds to the transcription factor JunD, resulting in the repression of JunD-induced transcription. We report here a detailed characterization of the zebrafish *men1* gene and its full-length (2551 nt) transcript, encoding a 617-amino acid protein with 67% identity and 80% similarity to human menin. Of the 81 missense mutations and in-frame deletions reported in MEN1 patients, 72 occur in residues that are identical in zebrafish, suggesting the importance of the conserved regions. The zebrafish *men1* gene maps 61 cM from the top of linkage group 7 (LG7), a region that appears to show conserved synteny to the *MEN1* loci at human 11q13. A 2.7-kb *men1* message is detected at all stages of zebrafish development analyzed, from one-cell embryos to adult fish. Whole-mount *in situ* hybridization showed ubiquitous distribution of *men1* message in zebrafish embryos at cleavage, blastula, gastrula, and early segmentation stages, with relatively abundant expression in blood cell progenitors (24 h post fertilization) and mesenchymal tissues (48 h post fertilization) at later stages. Zebrafish menin binds both human and mouse JunD, and represses JunD-induced transcription, indicating that the JunD-binding ability of menin is evolutionarily conserved.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a familial cancer syndrome characterized primarily by multiple tumors of the parathyroids and anterior pituitary, and entero-pancreatic endocrine tissues. Other tumors of the neuroendocrine (foregut carcinoids) and nonendocrine tissues (lipoma, angiofibroma, and collagenoma) are also associated with MEN1 (Metz et al. 1994). The *MEN1* locus was mapped to Chromosome (Chr) 11q13 by linkage studies (Larsson et al. 1988), and the responsible gene (*MEN1*) was identified by positional cloning (Chandrasekharappa et al. 1997). In addition to the germline mutations in nearly all MEN1 kindreds, somatic mutations in both the *MEN1* alleles in certain fractions of isolated parathyroid adenoma, insulinoma, gastrinoma, and lung carcinoids have also been observed (see Marx et al. 1999a). Among the nearly 250 independent mutations, spread throughout the coding region of *MEN1*, missense mutations and in-frame deletions account for about 30%, while the remaining are frameshift mutations leading to a truncated protein product, if expressed. Thus, *MEN1* appears to be a typical tumor suppressor gene whose inactivation results in the development of tumors. Despite the identification of a large number of mutations, no clear genotype-phenotype correlation has yet been discerned.

The human *MEN1* gene consists of 10 exons (the first being untranslated), encoding a 2.8-kb message and a 610-amino acid protein, termed menin. The amino acid sequence of menin exhibits no apparent similarities to any other known protein. Menin is primarily localized to the nucleus (Guru et al. 1998). In mouse NIH3T3 cells, the amount of menin appears to remain unaltered at G0, G1, and M stages of the cell cycle and log phase cells (Guru et al. 1999). Even though the process of tumorigenesis associated with the inactivation of menin remains to be elucidated, its binding to an AP1 transcription factor, JunD, and the resultant repression of transcription suggest a role for menin in transcriptional regulation (Agarwal et al. 1999).

Zebrafish has become an excellent model for developmental and genetic study of vertebrate development. In order to explore the functional role of this putative tumor suppressor during development, we have isolated and characterized the highly homologous zebrafish *men1* gene. The expression pattern, chromosomal location, and binding of zebrafish menin to human/mouse JunD (and resulting transcriptional repression) indicate that this is a highly conserved zebrafish ortholog of *MEN1*.

Materials and methods

Isolation and characterization of zebrafish men1 *cDNA and genomic clones.* A 5′-STRETCH plus cDNA library in lambda gt11 vector (Clontech, Palo Alto, CA), prepared from 1-month-old zebrafish (*Danio rerio*) by a combination of oligo-dT and random priming, was plated at a density of 50,000 pfu per 150-mm plate. In total, 0.5×10^6 plaques on replica filters were hybridized with ^{32}P-labeled probe. The probe included 325-bp and 311-bp PCR-amplified fragments, representing most of the exons 2–4 (nt 492–816) and the coding portion of exon 10 (nt 1594–1904) of the human *MEN1* cDNA sequence (Genbank accession no. U93236). Hybridization was performed overnight at 65°C in 6×SSC, 5×Denhardt's solution, 0.5% SDS, and the filters were washed at 55°C in 2×SSC, 0.1% SDS. Six clones were identified, and their inserts were released by *Eco*RI and *Not*I digestion and subcloned into pBSIIKS (Stratagene). The inserts from all the clones were sequenced, and, particularly, both strands of the large clone Z10 with 2.54 kb insert were completely sequenced.

For 5′-RACE reaction, an oligo-dT primed RT template was prepared from total RNA isolated from 3-day-old zebrafish embryos. RNA was isolated by the Trizol method (Life Technologies, Rockville, MD), and RT

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reaction was carried out with 4 μg RNA using reagents from the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA). Initial PCR was with primers ZF106 (TCAACAAAACCCAACACTAGAGACAGC) in exon 2 and AP1 (25 cycles), followed by a second PCR with nested primers AF105 (AGACTCGAAAAGCTGCACCACTCCATC) and AP2. The PCR products were cleaned using Geneclean kit (BIOS 101), ligated into a TA cloning vector (Invitrogen, Carlsbad, CA). Colonies positive by PCR for an internal primer pair were isolated and sequenced.

A zebrafish BAC library on high-density colony gridded filters (Genome Systems, St. Louis, MO) was hybridized with a 274-bp zebrafish *men1* cDNA probe, generated with primers ZF3 (GTCTCAGCCGATCATACTTCAA) and ZF10 (CTCCTTTCATTGATTCCAGCACT). The size of the BAC clone (b177b17) insert was determined by digestion with *Not*I, followed by PFGE (pulsed-field gel electrophoresis). For Southern analysis, the BAC DNA digested with *Eco*R1 was hybridized with a labeled full-length zebrafish *men1* cDNA as described above. Three hybridizing *Eco*R1 fragments (12-, 11- and 3.5 kb) were cut out from gel and subcloned into pBSIIKS (Stratagene, La Jolla, CA). Primers designed from the zebrafish *men1* cDNA sequence were used for sequencing the genomic clones. The sizes of larger introns were determined by long-range PCR using (flanking) exon-specific primers and reagents from the XL PCR Kit (Perkin-Elmer, Foster City, CA).

Northern blot analysis. Total RNA was extracted from different stages of zebrafish embryos and an adult male fish by Trizol method (Life Technologies). RNA samples (10 μg) were run on a 1% agarose-formaldehyde gel under denaturing conditions and stained with ethidium bromide to visualize the 28S and 18S rRNAs. Following transfer onto Hybond-N membrane (Amersham), filters were exposed to UV light for 1 min and hybridized to a labeled zebrafish *men1* cDNA probe (1070-bp insert from the Z4 cDNA clone). Hybridization was carried out at 42°C overnight in 50% formamide, 2×SSPE, 5×Denhardt's solution, 1% SDS, 100 μg/ml salmon sperm DNA, and filters were washed at high stringency in 0.1×SSPE, 0.1% SDS at 65°C.

Whole-mount in situ *hybridization.* Whole-mount *in situ* hybridization was performed using digoxigenin-labeled, full-length zebrafish *men1* probes as described (Hauptmann 1994). Sense and antisense digoxigenin-labeled RNA probes were prepared by *in vitro* transcription from linearized templates of full-length zebrafish *men1* cDNA clone (Z10) in the pBS vector. The sense control probe did not produce a hybridization signal (data not shown). For sectioning, specimens were embedded with reagents from JB-4 plus embedding kit (Polysciences, Inc., Warrington, PA), sectioned at 5-μm thickness and counterstained with Eosin.

Mapping by RH analysis. The T51 radiation-reduced zebrafish/hamster hybrid panel (Research Genetics, Huntsville, AL) was used to map *men1* by PCR (Geisler et al. 1999) with primers ZF32 (GAGGCATCGATGGAGTGGT) and ZF34 (TGTTGACGGCCAGGAAGTGC). The PCR results were submitted online (*http://www.eb.tubingen.mpg.de/abt.3/hafter lab/rh mapping. html*) to locate the *men1* gene on the radiation hybrid map of the zebrafish genome.

In vitro *translation (IVT) and GST-JunD binding assay.* Plasmid DNAs containing full-length *MEN1* cDNAs from human (A11) and zebrafish (Z10) were *in vitro* transcribed/translated in the presence of 35S-methionine with the rabbit reticulocyte system (Promega, Madison, WI) according to the supplier's protocol. Expression of GST and GST-JD1 (JD1 = amino acids 8–340 of human JunD) in bacteria, and their attachment to agarose-glutathione, s well as the conditions for the GST Pull-Down assay were as described earlier (Agarwal et al. 1999). Briefly, GST-JD1 or GST attached to agarose beads were incubated with 10 μl of IVT menin in the binding buffer (50 mM Tris-HCl [pH8], 100 mM NaCl, 0.3 mM DTT, 10 mM MgCl2, 10% glycerol, and 0.1% NP40, 2% BSA with protease inhibitors). The beads were washed three times with the same buffer, suspended in 20-μl sample–SDS buffer, and run on a 10–20% gradient gel. The retention of 35S-labeled menin was identified by autoradiography.

Effect of zebrafish menin on JunD transactivation. The effect of zebrafish menin on JunD-induced transcription was studied in 293wt cells as described earlier (Agarwal et al. 1999). Briefly, cells were seeded 24 h prior to transfection in six-well plates. For determining the effect of menin on Gal4 reporter-mediated activation by Gal4DBD-JD1 fusion protein

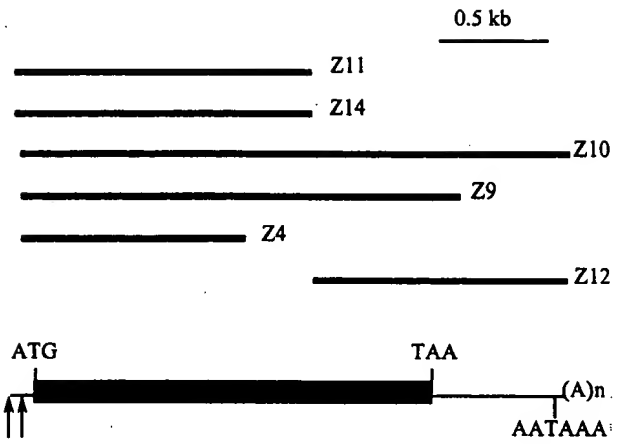

Fig. 1. Isolation of zebrafish *men1* cDNA. The extent of overlap among the six clones (Z11, Z14, Z10, Z9, Z4, and Z12) isolated by screening a zebrafish cDNA library with human *MEN1* cDNA probe is shown. The clone Z10 covers the entire transcript except for the 11 nt at the 5′ end. The full-length cDNA sequence (2551 nt) assembled from these overlapping clones is shown below. A filled rectangular box with the initiation (ATG) and termination (TAA) codons shows the ORF of 617 amino acids, flanked by 92-nt 5′UTR and 605-nt 3′UTR. Arrows point to two potential start sites, and the polyA signal (AATAAA) at −22 is also indicated.

(JD1 = amino acids 8–340 of human JunD), cells were transfected with 0.5 μg Gal4DBD-JD1, and increasing amounts of human or zebrafish menin expression plasmid (0.3 μg and 2 μg) along with 0.5 μg reporter plasmid pFR. pFR contains five copies of the Gal4 UAS upstream of a minimal TATA and luciferase reporter gene. For determining the effect of menin on AP1 reporter-mediated activation by JunD, cells were transfected with 0.5 μg pcDNA3.1 mouse JunD (mouse JunD expression plasmid) and increasing amounts of H-menin or zebrafish-menin expression plasmid (0.3 μg and 1 μg), along with 0.5 μg reporter plasmid pAP1.pAP1 contains seven copies of the AP1 consensus TRE 'TGACTAA' upstream of a minimal TATA and luciferase reporter gene. The same amount of total DNA transfected was maintained in all transfections by adding the empty vector pcDNA3.1. Luciferase assays were performed 48 h after transfection by using Luciferase assay system (Promega). Cell lysates and assays were performed as per the product manual supplied by the manufacturer. All results are expressed for equal amounts of protein. To assess the expression of transfected menin and JunD, Western blot analysis was performed with the above cell lysates by using an anti-menin antibody (GPN) and an anti-JunD antibody (Santa Cruz Biotechnology). The GPN antibody is directed against a peptide corresponding to amino acids 187–211 (GPNGEQTAEVTWHGKGNEDRRGQTVA) of human menin.

RESULTS

Isolation and characterization of zebrafish men1 *cDNA.* Screening a zebrafish cDNA phage library with two labeled fragments representing exons 2–4 and 10 of the human *MEN1* cDNA as probes identified six clones. The sizes of the inserts in these six overlapping clones, Z4, Z9, Z10, Z11, Z12, and Z14, ranged from 1.1 kb to 2.5 kb (Fig. 1). The inserts released by *Not*I/*Eco*RI digestion of the phage clones were subcloned into pBSIIKS plasmid and sequenced. Clones Z10, Z9, and Z4 start at the same point but extend up to 2540 nt, 2010 nt, and 1070 nt respectively. Clone Z10 has 81-nt 5′-UTR, 617-amino acid open reading frame (ORF), and terminates in a polyA tail at the 3′-end with a polyA signal at −22. The identical size (1305 bp) inserts in clones Z11 and Z14, probably sibs of a single clone, extend the 5′ end further by 11 nt. Therefore, two start sites, at 11-nt apart, may be operating in transcription of the zebrafish *men1*. 5′ RACE results did not extend the 5′-end any further, but did generate two groups of clones supporting both the start sites. The clone Z12 starts near the middle at nt 1310 and extends to the 3′ end. The full-length 2551-nt zebra-

(A)

Exon/intron	Exon length (bp)	Starting position in cDNA	Human Exon length (bp)	Acceptor splice site	Donor splice site	Intron length (bp)
1	54	1	81	----------	AACAAAG/gtatgtt	~4, 000
2	474	55	474	ttctttttatttttag/GCTACAT	ATTACAG/gtaaagt	~4, 000
3	209	529	209	atttttgttggtaag/GCACTAA	TGAAAGG/gtaatgc	362
4	129	738	129	tttccatttcattag/AGCTGGT	CCAGCAA/gtatgtc	308
5	41	867	41	atttctcttttgcag/AGGCTGT	TGGAAAG/gtgtgat	661
6	88	908	88	cttggtgtgttacag/GTATCCT	CCTTAAG/gtatttg	91
7	137	996	137	ttttgtatttcccag/GCTGTTG	TGCAAGA/gtaagtt	~ 5, 000
8	133	1,133	133	ttatttttctttacag/TTACAAC	AAAAGAG/gtcttgt	111
9	174	1,266	174	tatttccctcaaaag/GACCAGC	TGCACAG/gtgtaaa	1,162
10	1,112	1,440	1,276	tatttatctttctcag/ATCAGGC	----------	

(B)

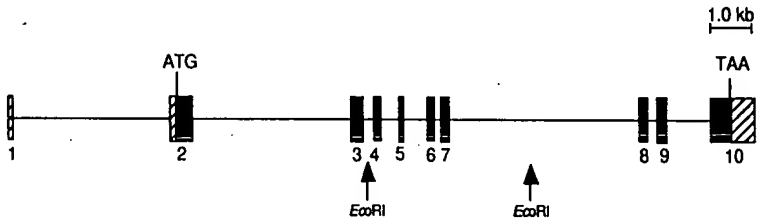

Fig. 2. The exon-intron organization of the zebrafish *men1*. **(A)** Comparison of cDNA and genomic DNA sequence showed that the gene consists of 10 exons. The lengths of exons and introns, and the location of each exon are given. For comparison, exon lengths of human *MEN1* (shaded) are also given. The sequences at the exon-intron splice junctions follow the gt—ag rule. **(B)** A schematic representation of the organization of the exons (1–10, rectangular boxes) and introns (thin lines). The noncoding regions, exon 1 and parts of exon 2 and 10, are indicated by hatched boxes. The locations of two *Eco*RI sites are also shown.

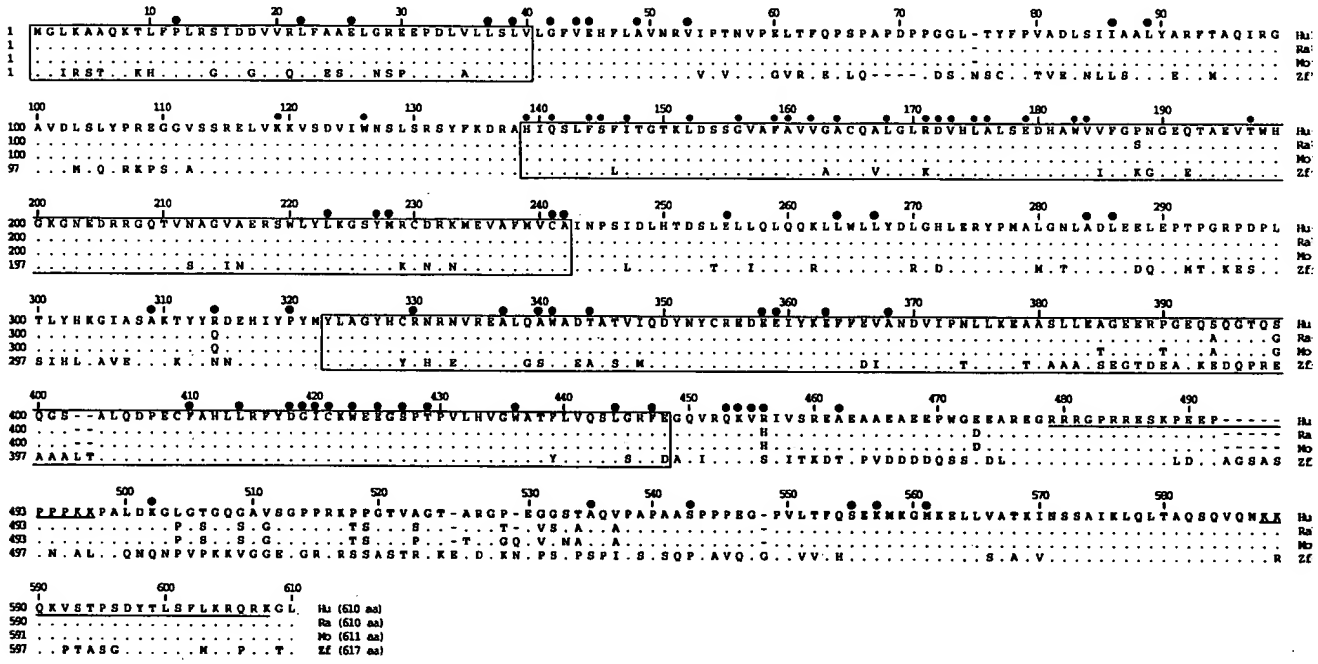

Fig. 3. Alignment of menin sequences. The amino acid sequences (in single letter codes) of menin from human (Hu), rat (Ra), mouse (Mo), and zebrafish (Zf) are compared by using the DNASTAR Megalign program. The amino acid residues identical to the human sequence are indicated by a dot. The filled circles on top of the human sequence indicate the 81 amino acids that are affected by 61 missense mutations and 22 in-frame deletions (two amino acids are affected by both mutations). The boxes around aa 1–40, 139–242, and 323–448 identify the three regions in human menin critical for JunD binding (Agarwal et al. 1999). The nuclear localization signals in human menin, NLS1 (aa 479–497) and NLS2 (aa 588–608), are underlined.

fish *men1* cDNA sequence (Genbank accession no. AF212919) contains a 92-nt 5′ UTR and a 605-nt 3′ UTR, in addition to the ORF encoding a 617-amino acid protein that shares 67% identity and 80% similarity with the human menin.

Genomic structure of zebrafish men1. In order to determine the genomic structure of *men1*, a BAC library was screened by hybridization with a labeled *men1* cDNA probe. A 100-kb BAC clone (b177b17) was identified that contained the entire gene as determined by PCR amplification of both ends of the zebrafish gene. Southern hybridization of *Eco*R1 digests of the BAC DNA with the full-length *men1* cDNA probe identified three fragments, 3.5, 10, and 12 kb. These fragments were isolated, subcloned, and sequenced with primers designed based on the cDNA sequence. Comparison of the genomic and cDNA sequences helped determine exon-intron organization. The 3.5-kb subclone, and the flanking 10-kb (5′-) and 12-kb (3′-) clones contained the entire gene. The number of exons and even the sizes of exons 2–7 are identical to those of human gene. However, introns 1, 2, and 7 were found to be significantly larger. Their sizes were determined by long-range PCR with (flanking) exon-specific primers on genomic DNA templates. The large size of introns 1 and 2 (~4 kb each) and 7 (~5 kb) accounts for the bigger size of zebrafish *men1* gene compared with human (18.2 kb *vs* 7.2 kb). The splice donor and

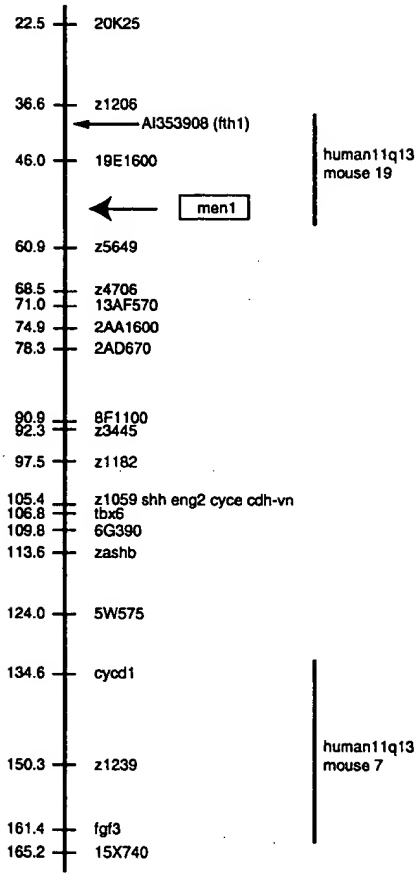

Fig. 4. The zebrafish *men1* maps to the linkage group 7 (LG7). A RAPD marker-based meiotic map of LG7 (*http://zebrafish.mgh.harvard.edu/map comparison/LG07.html*) shows the location of *men1*. The numbers to the left of each marker indicate the distance in cM from top of the chromosome. RH mapping localized *men1* to 21cR (~1.3 Mb) from marker z5649. Location of zebrafish *fth1* near *men1* is also indicated. Two regions of LG7 sharing the genes from human 11q13, but from two different mouse chromosomes (19 and 7), are indicated.

acceptor sites are in agreement with the consensus sequences (Fig. 2).

Comparison of human, mouse, rat, and zebrafish menin sequences. Mouse and rat homologs of *MEN1* have now been isolated and characterized (Bassett et al. 1999; Guru et al. 1999; Karges et al. 1999; Stewart et al. 1998). The amino acid sequence of the zebrafish menin (AF212919) was compared with the sequences from human (U93236), rat (AB023400), and mouse (AF109389) (Fig. 3). The protein sequences are highly conserved through evolution. Zebrafish, mouse, and rat menin share 67%, 96.7%, and 97.2% identity with human menin, respectively. Similarity between the human and zebrafish menins is as high as 80%. Among the 81 amino acids that are altered owing to disease-causing missense mutations and in-frame deletions, 54/61 missense mutations and 20/22 in-frame deletions are in identical amino acid residues in zebrafish. That this is significantly more than the overall 67% identity indicates the importance of these evolutionarily conserved residues (Fig. 3). Apart from an in-frame deletion, V44del (Cebrian et al. 1999), and two missense mutations, L444P (Cetani et al. 1999) and A462P (Farnebo et al. 1999), all the 81 mutations are summarized in Marx et al. (1999b).

Both of the nuclear localization signals, NLS1 and NLS2, are generally conserved in zebrafish menin. In human, the basic residues (in bold) at both NLS1 (**RRRGPRRESKPEEPPPPKK**) and NLS2 (**KKQKVSTPSDYTLSFLKRQRK**) could allow them to function either as a single sequence or bipartite type signal (Fig. 3). Although both NLSs in zebrafish could provide a single sequence type signal, only NLS2 sequences could function as a bipartite type signal.

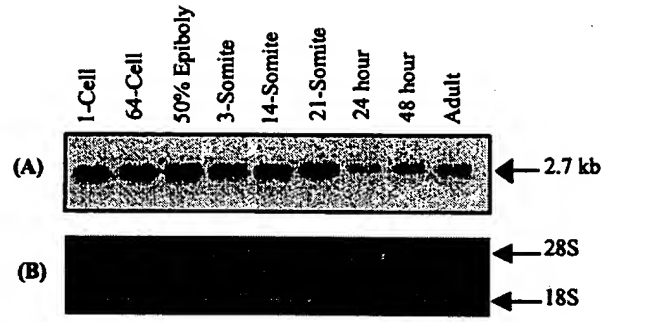

Fig. 5. Northern analysis. **(A)** Hybridization of a zebrafish *men1* cDNA probe to a Northern blot containing RNA isolated from various stages of zebrafish development (indicated on top). A single band of 2.7 kb is observed in all the lanes. **(B)** Ethidium bromide-stained gel before transfer shows the ribosomal RNA bands that serve as loading controls.

Mapping of the zebrafish men1 *gene to LG7 by RRH.* Radiation hybrid mapping indicates that the zebrafish *men1* is located on linkage group 7 (LG7), 21 cR (approximately 1280 kb) from marker z5649 located at 61 cM from the top of the chromosome (Fig. 4). This region of LG7 contains the FTH1 homolog (J. Yoder et al. in preparation), which is also present on human (11q13) and mouse (19 B-C2), suggesting the conserved synteny of the *MEN1* loci among human, mouse, and zebrafish chromosomes. In addition, zebrafish homologs of two genes, APJ (angiotensin G-protein coupled receptor-like) and Factor II (prothrombin), known to map proximal to *MEN1* on human 11q13, also map near zebrafish *men1* on LG7 (A. Vogel, unpublished results), supporting the conserved synteny of this region in human and zebrafish genomes.

Expression analysis by Northern and in situ *hybridization.* Northern blot analysis of RNA (Fig. 5) isolated from different stages of development showed a single band of 2.7 kb in all the samples tested, from one-cell embryos to adult fish. The presence of the *men1* message in one-cell stage embryos suggests that it is expressed maternally. The *men1* expression was observed at all the stages tested.

The embryonic expression pattern of zebrafish *men1* was investigated by whole-mount *in situ* hybridization. As expected from the Northern hybridization results, *men1* transcripts were detected at all stages. From cleavage through somitogenesis stages, *men1* transcripts appear to be uniformly distributed throughout the embryo (data not shown). At the end of somitogenesis (24 h post fertilization [h.p.f.]), prominent staining is detected in the trunk in the Intermediate Cell Mass (ICM), the site of primitive hematopoiesis in fish (Fig. 6A, C, D). Histological sections revealed that the staining is concentrated in the cytoplasm of the large, round ICM proerythroblasts (Fig. 6C). In addition to strong expression in maturing blood cells, *men1* expression is also detected in the central nervous system, including the spinal cord (Fig. 6A–E and data not shown), and in head and fin mesenchyme. We also examined the expression of *men1* in two bloodless mutants, *vampire*^{m262} (*vmp*) and *vlad tepes*^{m651} (*vlt*) (Weinstein et al. 1996). Both mutants lack virtually all blood cells at the end of somitogenesis, and strong expression of *men1* in ICM proerythroblasts is absent. However, weak *men1* expression in the vicinity of the ICM was still observed in mutants, in the ventromedial mesenchyme (Fig. 6A, B, E). In whole-mounted, wild-type embryos this staining is obscured by the strong stain of the blood cells, but is visible upon close inspection of sections (data not shown).

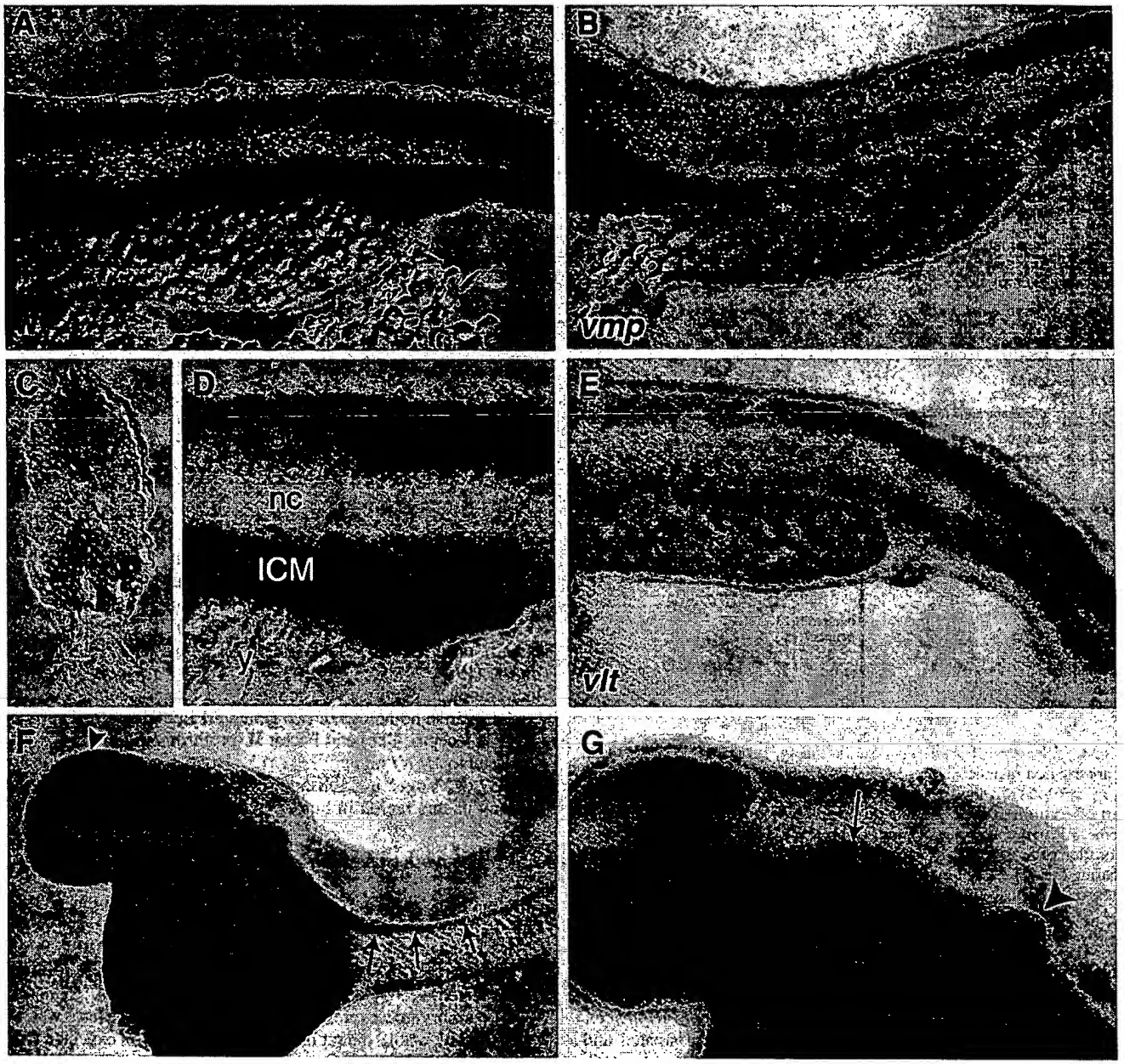

Fig. 6. Embryonic expression pattern of *men1*. **A–E:** 24 h.p.f. *men1* expression is detected in the ICM hematopoietic progenitors, neural tube, and ventral mesenchyme of the trunk and tail (see **D** for identification of anatomical structures). The hematopoietic expression is absent in the bloodless mutants *vampire* (*vmp*, **B**) and *vlad tepes* (*vlt*, **E**), while the expression in other tissues is unaffected. **C**, transverse section at the anal level: large round proerythroblasts ventral to the somites express *men1*. **D** shows a magnification of a whole mount *in situ hybridization* of the same region as **C**, same scale. sc, spinal cord; nc, notochord; ICM, intermediate cell mass; y, yolk extension. **F, G:** 48 h.p.f.: *men1* expression is detected in the tectum (arrowhead in **F**), endoderm (arrows in **F**), eye (**G**), otic vesicle (arrow in **G**), fin bud (arrowhead in **G**), and branchial arches (bracket in **G**). All except **C** are lateral views, anterior to the left.

At 48 h post fertilization, expression is not apparent in blood, but is still weakly detected in ventral trunk mesoderm (Fig. 6F). Expression is also detected in the endoderm, including the liver primordium (Fig. 6F and data not shown), as well as in the retina, tectum, otic vesicle, fin bud mesenchyme, and branchial arches (Fig. 6G).

Zebrafish menin binds mammalian JunD and inhibits JunD-induced transcription. Both the zebrafish and human menin were tested for their binding to human JunD by using a previously described GST pull-down assay (Agarwal et al. 1999). As evident from Fig. 7A, JunD retains zebrafish menin, as it does human menin, indicating that the JunD binding characteristics of menin are evolutionarily conserved.

To investigate the effect of zebrafish menin on JunD-activated transcription, 293wt cells were co-transfected with Gal4DBD-JD1 and a Gal4 reporter (pFR; Stratagene), along with human menin or zebrafish menin expression plasmid. The pFR plasmid contains five copies of the Gal4 UAS upstream of a minimal TATA and luciferase as reporter. The transactivation by JunD was repressed effectively by zebrafish menin in 293wt cells (Fig. 7B). Control experiments, co-transfecting Gal4 reporter along with Gal4DBD-JD1, and empty vector (pCMVsport) did not effect the Gal4DBD-JD1-mediated luciferase activity.

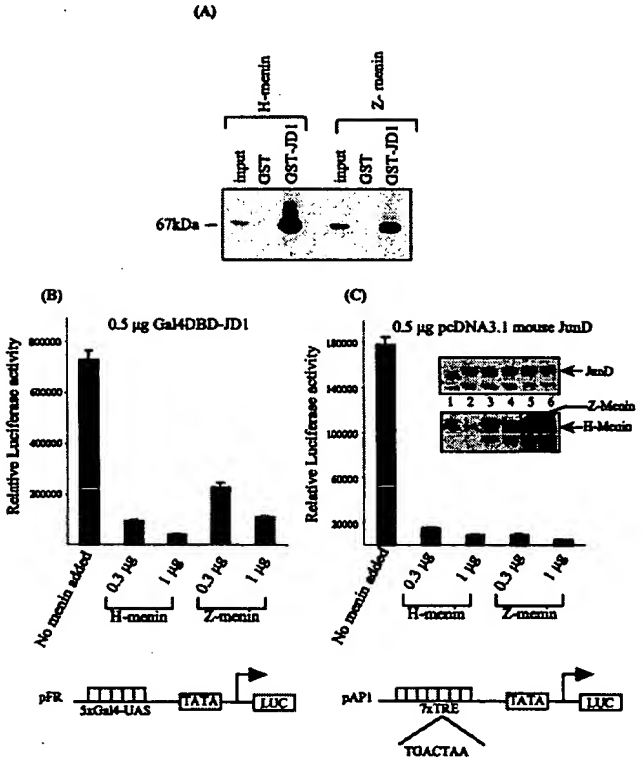

Fig. 7. Zebrafish Menin binds JunD and represses its transcriptional activity in 293wt cells. **(A)** Retention of labeled IVT human menin (H-menin) and zebrafish menin (Z-menin) by GST-JD1 (human JunD), but not by GST. Input lane shows a tenth of the sample used in the binding assay. **(B)** Menin represses transactivation by Gal4DBD-JD1 fusion protein. The luciferase reporter plasmid pFR (0.5 μg) was co-transfected into 293wt cells with Gal4DBD alone or Gal4DBD-JD1 plasmid, and various amounts of H-menin or Z-menin expression plasmid (0.3 μg, 1 μg) or empty vector. The empty vector pcDNA3.1 was added to maintain equal amount of DNA transfected. Results are expressed as relative luciferase activity. Empty vector without menin insert had no effect on Gal4DBD-JD1-induced transcription (data not shown). Minimal reporter activity was observed with Gal4DBD alone (data not shown). Results from a representative experiment performed in triplicate, with mean and standard error, is shown. Below each graph are diagrams of the reporters used. **(C)** Z-menin represses transactivation by JunD from a TRE containing reporter plasmid. 293wt cells were transiently transfected with 0.5 μg of the luciferase reporter plasmid pAP1, 0.5 μg of pcDNA3.1mouseJunD (mouse JunD expression plasmid), and various amounts of H-menin or Z-menin expression plasmid (0.3 μg, 1 μg), or empty vector. The empty vector pcDNA3.1 was added to maintain equal amount of DNA transfected. Results are expressed as relative luciferase activity. Empty vector without menin insert had no effect on JunD induced transcription (data not shown). Minimal reporter activity was observed with the menin expression plasmid pCMV sportMenin alone (data not shown). Levels of expression of menin and JunD in this experiment are shown below the diagram of the reporter. Equal amounts (30 μg) of total cell lysates were analyzed by Western blot analysis by using anti-menin antibody (GPN) or anti-JunD antibody. Lane 1: untransfected cell lysate; lane 2: JunD alone; JunD was co-transfected with H-menin or Z-menin, in lanes 3 to 6: Z-menin 0.3 μg, 1 μg, H-menin 0.3 μg, 1 μg.

To examine the consequences of zebrafish menin on AP1-mediated JunD transcriptional activation, increasing amounts of zebrafish menin expression construct were co-transfected into 293wt cells, with mouse JunD expression plasmid, and an AP1 reporter plasmid (pAP1; Stratagene). pAP1 contains seven copies of TGACTAA as the consensus TRE upstream of a minimal TATA and luciferase as reporter. Similar to the results obtained above with Gal4 reporter, the transactivation by JunD was repressed effectively by zebrafish menin (Fig. 7C). This activity was not repressed when menin expression construct was substituted by empty vector (pCMVsport) (data not shown).

Western blot analysis confirmed the expression of transfected zebrafish menin in cell lysates used for reporter assays (Fig. 7C). The anti-menin antibody GPN is directed against a peptide spanning amino acids 187–211 of human menin, which has 88% identity in the corresponding region of zebrafish menin. As evident from the blot, the GPN antibody shows weak interaction with zebrafish menin, but does demonstrate that the repression is associated with the expression of zebrafish menin in the transfected cells.

Discussion

The amino acid sequence of the *MEN1* gene-encoded protein, menin, has not revealed any clues as to its function. Interspecies sequence comparisons of menins may identify functionally significant domains that should be highly conserved among species. We and others have isolated and characterized the mouse homolog of the *MEN1* gene from the syntenic region on mouse Chr 19 (Bassett et al. 1999; Guru et al. 1999; Karges et al. 1999; Stewart et al. 1998). A rat homolog has also been identified and characterized (Karges et al. 1999), and an independent sequence entry for the rat *Men1* is also available (Genbank accession no. AB023400).

We have now isolated a full-length zebrafish *men1* cDNA (Genbank accession no. AF212919) that encodes a 617-aa polypeptide, slightly longer than the human, rat (610 aa), and mouse (611 aa) proteins (Fig. 3). The start codon in zebrafish (accATGg) matches well with the "Kozak consensus," as do the start codons for the other three genes (gccATGg). The zebrafish cDNA has a typical poly A signal AATAAA at −22 from the polyA tail. The polyA signals in human (AATACA), and mouse/rat (GATAAA) are at −13 and at −19 respectively. While this manuscript was in preparation, a report of the zebrafish *men1* sequence, determined from a clone with an EST (AI794424) matching human *MEN1* sequence (Khodaei et al. 1999), appeared. The 5′ end of this zebrafish sequence (AJ245952) matches our second start site, and two nucleotide changes in the coding region encode different amino acids at 361 and 369 from conserved residues in human, rat, mouse, and our zebrafish sequence. It is not clear whether these represent polymorphisms or possible sequencing errors. In addition to the cDNA sequence, the genomic organization, expression, and functional analysis reported here should serve as useful tools to explore the biology of menin.

The nucleotide sequence of the zebrafish *men1* coding region is 67% identical to the corresponding human sequence. Comparison of the menin sequences from human, mouse, rat, and zebrafish indicates a high sequence conservation across these species (Fig. 3). The importance of this conservation is made even more meaningful by the fact that 89% of the missense mutations and 91% of the in-frame deletions in human patients occur in amino acid residues that are identical in zebrafish. Mouse and rat menins show 96–100% identity with human menin, except for a 35-amino acid region (aa 504–538) that exhibits ~60% identity. A comparable region in zebrafish menin (aa 508–543) also differs significantly, with only 7/36 residues being identical to human. In zebrafish, this region of high divergence extends an additional 18–20 amino acids in either direction. Except for a small stretch of about 20 amino acids (residues 380–401), amino acids 110–454 in zebrafish menin are very similar to the corresponding human/rat/mouse sequences.

Zebrafish *men1* maps to linkage group 7 (LG 7), a region of conserved synteny with the *MEN1* locus on human 11q13, as three other genes are shared by these two regions, *FTH1*, *APJ*, and *FII*. Other genes, including *Cycd1* and *Fgf3*, are shared between 11q13 and LG7, but their synteny is not conserved along LG7, as genes orthologous to 11p12 (*spi1*), 7q36 (*shh* and *eng2*), and 19q12 (*cyce*) are found in between *men1* and *cycd1* in zebrafish. In the

mouse, *Fth1* and *Men1* are found on mouse Chr 19, whereas *Ccnd1* (zebrafish *cycd1*) and *Fgf3* are found on mouse Chr 7. Thus, zebrafish LG7 has two different regions of synteny for the 11q13 genes (Fig. 4).

Northern analysis shows that zebrafish *men1* is expressed as a single 2.7-kb mRNA species. This is unlike mouse, where variable inclusion of intron 1 results in two equivalent messages with the 5′ UTR differing by 382 nt. Analysis of the comparable 5′ UTR region by PCR has identified similar transcripts with alternative splicing in rat and human transcripts (Karges et al. 1999). Ubiquitous expression was observed throughout early development of the zebrafish, up to early somitogenesis, by whole-mount *in situ* hybridization. By 24 hpf localized expression was seen in maturing blood cells, with lower level specific expression in the central nervous system, endoderm, and a number of mesenchymal tissues including fin, head, and trunk mesenchyme. It is not known at present whether mouse *Men1* is similarly expressed in yolk sac hematopoietic progenitors at comparable stages. However, expression analysis in various adult (8–12 weeks old) and fetal (18 day pc) mouse tissues by quantitative PCR has shown increased expression in hematopoietic and neuroendocrine tissues (Karges et al. 1999), resembling to some extent the enhanced expression of maturing blood cells in zebrafish. In 48 hpf zebrafish, *men1* is expressed in the eye, midbrain, and limb buds. At a similar developmental stage (10.5 and 11.5 days p.c.), mouse embryos show enhanced expression of *Men1* in the homologous tissues (Guru et al. 1999). Zebrafish *men1* expression at 48 hpf also shows a good correlation with the expression in adult mouse tissues, as analyzed by Northern analysis. Both mouse and zebrafish *men1* genes are detected in the brain and liver. The endodermally derived mouse lung, which expresses *Men1*, does not have a homologous structure in fish, but zebrafish *men1* is expressed in endoderm. In both species, *Men1* is not expressed in skeletal muscle at a high level. However, there is strong expression of mouse *Men1* in kidney and testis, while the zebrafish gene is not expressed in the kidney. Since zebrafish embryos have a pronephric kidney and the adult mouse has metanephric kidney, these organs may not be fully equivalent. These patterns of expression in the zebrafish do not, however, shed obvious light on the reasons for prominence of endocrine tumors in human MEN1.

Binding to JunD and associated transcriptional repression suggest that zebrafish *men1* is a true functional ortholog of human *MEN1*. Zebrafish *junD* has not yet been identified. Three regions in human menin (amino acids 1–40, 139–242, and 323–448) were shown earlier to be critical in JunD binding (Agarwal et al. 1999). All three homologous regions in zebrafish share an extensive homology with human menin, the 1–40 aa and 139–242 aa showing the least (68%) and the most (91%) conserved residues, respectively.

Survey of the existing collection of mapped developmental mutants does not reveal an obvious candidate for a phenotype that might be associated with mutations in zebrafish *men1* (http://zfish.uoregon.edu/). An important future priority to explore will be the development of such a model, either by additional mapping of ENU-induced mutants, insertional mutagenesis, or the use of antisense technology.

Acknowledgments. We thank Drs. Jeffrey Yoder for the information on zebrafish *fih1* mapping, Paul Goldsmith for GPN antibodies, and Brian Pike for help in *men1* mapping using a zebrafish RH panel. A.M. Vogel was supported by a postdoctoral fellowship from DAAD (Gemeinsames Hochschulsonderprogramm III von Bund und Ländern). T. Oda was supported by a fellowship from the Japanese Society for the Promotion of Science.

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Int. J. Cancer: **85**, 877–881 (2000)

Publication of the International Union Against Cancer

EXPRESSION ANALYSIS OF ENDOGENOUS MENIN, THE PRODUCT OF THE MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 GENE, IN CELL LINES AND HUMAN TISSUES

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We have investigated the endogenous expression of menin, a protein encoded by the gene mutated in multiple endocrine neoplasia type 1 (MEN1). Western blot analysis showed strong expression of menin as a 68 kDa protein in all of 7 human and primate cell lines tested. In a panel of 12 fetal human tissue extracts, 68 kDa menin was readily detected in brain cortex, kidney, pituitary, testis and thymus and weakly detected in thyroid. Reproducible bands other than 68 kDa were observed in adrenal and heart, whereas menin was undetectable in liver, lung, pancreas and skin. Analysis of synchronized HeLa cells revealed no variation in the amount or size of menin throughout the cell cycle. Protein expression was compared between lymphoblastoid cell lines from healthy controls and MEN1 patients carrying nonsense mutations on 1 allele. No truncated protein was detected in either cytoplasmic or nuclear fractions in mutation-carrying cells. The expression level and cellular location of full-length menin did not differ between cell lines derived from MEN1 patients and healthy donors. This suggests that the wild-type allele has been up-regulated in mutation-carrying cells to compensate for the loss of 1 functional allele. *Int. J. Cancer* 85: 877–881, 2000.

Multiple endocrine neoplasia type 1 (MEN1) (MIM: 131100) is a hereditary syndrome transmitted as an autosomal dominant trait. It is characterized by multiple occurrence of tumors of the parathyroids, endocrine pancreas and anterior pituitary (Wermer, 1954). Other endocrine neoplasms, such as adrenal tumors and carcinoids, and non-endocrine tumors, such as lipoma, are seen less frequently (Trump *et al.*, 1996). The disease locus was previously mapped to 11q13 near the PYGM marker (Larsson *et al.*, 1988), and the responsible gene has been identified (Chandrasekharappa *et al.*, 1997; European Consortium on MEN1, 1997). The *MEN1* gene covers a 9 kb genomic region and produces a widely expressed transcript of 3 kb. The open reading frame (ORF) is composed of 610 codons and is translated into a 68 kDa protein named menin. A direct interaction between menin and JunD has been demonstrated, and expression of wild-type menin can inhibit the transcriptional activity of JunD *in vitro* (Agarwal *et al.*, 1999).

To date, the identified *MEN1* mutations are mainly frameshift or nonsense mutations, which coincide with loss of heterozygosity in both familial and sporadic MEN1-associated tumors (Agarwal *et al.*, 1997; Bassett *et al.*, 1998; Giraud *et al.*, 1998). These patterns of inactivation strongly suggest that menin acts as a tumor suppressor. Known missense mutations are scattered over the entire coding region, which suggests that multiple domains of the protein are responsible for its biological function.

It has been shown, by both immuno-histochemical and subcellular fractionation methods, that menin is located mainly in the nucleus (Guru *et al.*, 1998). Two putative nuclear localization signals (NLSs) have been found in the C-terminal region by deletion mapping. About 80% of the known frameshift and nonsense mutations lead to a truncated peptide lacking both NLS1 and NLS2 motifs, whereas NLS2 is maintained in 20% of mutations. Although NLS1 alone appears to be sufficient for the nuclear localization of the GFP–menin fusion protein *in vitro*, little is known about the functionality of these NLSs *in vivo* or the effect of mutations on the cellular location.

Here, we report the detection of endogenously expressed menin in a panel of both human and primate cell lines and 12 human embryonic tissues at week 20 of gestation. We further investigate the expression and subcellular localization of menin during the cell cycle and the effect of truncating mutations on menin in lymphoblastoid cell lines from MEN1 patients.

MATERIAL AND METHODS

Cell culture, cell transfection and tissues

Adherent and non-adherent cells were maintained under standard conditions in DMEM and RPMI-1640, respectively, using 10% FCS. Cell lines used in our study include Cos-M6, Bosc 23 [a highly transfectable ectopic virus-packaging cell line (Pear *et al.*, 1993)], GDH (a glioma line), HeLa, PC12, U87 [a glioma line (Furnari *et al.*, 1997)] and 4 lymphoblastoid cell lines (LCLs) previously established in our laboratory (Giraud *et al.*, 1998). The lines LCL-F23M1 and LCL-F42M1 carry germ-line mutations R415X and R527X, respectively, while LCL-FN1 and LCL-FN2 are from non-affected individuals from a MEN1 family. Transfections were performed by calcium phosphate co-precipitation according to standard protocols with the following modifications: 24 hr before transfection, Bosc cells were plated at 7×10^6 cells/ml per culture in a 100 mm dish and 30 μg of plasmid DNA used. The efficiency of transfection was increased by treatment with chloroquine at 25 μM. A panel of 12 normal human tissues were obtained from aborted fetuses of gestation week 20, including adrenal, brain cortex, heart, kidney, liver, lung, pancreas, pituitary, skin, testis, thymus and thyroid.

Cell-cycle synchronization

HeLa cells were serum-starved (0.5% FCS) for 48 hr to obtain cells at G_0 phase and treated by double thymidine block with 25 mM thymidine (Sigma-Aldrich, Saint Quentin Fallavier, France) for 16 hr, with an 8 hr release interval, and tested at 0, 3 and 6 hr, to obtain cells synchronized, respectively, at the G_1/S, S and S/G_2 boundaries. To obtain mitotic cells, HeLa cells were directly treated with 400 ng/ml nocodazol (Sigma-Aldrich) and incubated for 16 hr. Synchronized cells were harvested, washed 3 times with cold culture medium and subjected to subcellular fractionation as described below. Flow-cytometric analysis was done using FACSCALIBUR 3CA (Becton Dickinson, Mountain View, CA).

Grant sponsor: Swedish Cancer Foundation and King Gustaf V Jubilee Foundation; Grant number: 98:528; Grant sponsor: l'Association pour la Recherche contre le Cancer; Grant numbers: 9211; PHRC 97-048; Grant sponsors: Ligue contre le Cancer de Saône-et-Loire; Ligue contre le Cancer du Rhône et de la Drôme.

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Received 26 June 1999; Revised 18 October 1999

Constructs

MEN1 cDNA was obtained from a human testis library (Stratagene, Saint Quentin en Yvelines, France). A tag sequence for the VSV-G epitope (YTDIEMNRLGK) was incorporated by PCR amplification of the 5′ end of the cDNA, using primers 5′-CCCCGCGGTATGTACACTGATATCGAAATGAACCGCCT-GGGTAAGGGGCTGAAGGCCGCCCA-3′ (cDNA position 111–130), and 5′-CCGTTACGGGAACACCATCTC-3′ (position 921–943). The complete cDNA encoding the N-tagged menin was subcloned into a NotI-cut pCI-neo expression vector (Promega, Charbonnières, France) and referred to as pCI-NtM1-S. The constructs for menin expression in bacteria were assembled by inserting a PCR-generated *MEN1* cDNA (position 146–2050) into SmaI-cut pQE30-32 vector (Qiagen, Courtaboeuf, France). An in-frame construct, pQE-FM1, was used; an out-of-frame insert, pQE-FM4, was used as a negative control.

Protein extract preparation and isolation of nuclei

Extracts from cell lines were prepared as follows: the medium was removed and cell layers were rinsed with PBS and lysed in a modified RIPA lysis buffer containing NaCl 150 mM, EDTA 0.2 mM, Tris-HCl (pH 7.5) 20 mM, NP40 1% and SDS 0.2% supplemented with a cocktail of protease inhibitors (1 mM PMSF; 10 μg/ml aprotinin, leupeptin and pepstatin; 1 mM orthovanadate). After 20 min incubation at 4°C, the lysate was centrifuged at 13,000 *g* for 15 min at 4°C to remove insoluble material. Frozen tissues were pulverized and lysed in the above solution. Protein concentration was determined using a modified Bradford analysis kit (Bio-Rad, Ivry sur Seine, France).

Cellular cytoplasmic and nuclear fractions were separated using lysis in Nonidet P-40 buffer. Cell pellets were suspended in a 0.2 M sucrose/3 mM $CaCl_2$/2 mM magnesium acetate/0.1 mM EDTA/10 mM Tris-HCl (pH 7.5)/1% Nonidet P-40 buffer and incubated at 4°C for 30 min. After microscopic examination to ensure that cell lysis occurred and that the nuclei appeared to remain intact, the nuclear pellet was sedimented. The cytoplasmic fraction was centrifuged to remove any trace of nuclei and the nuclear pellet suspended in the modified RIPA buffer. Subcellular nuclear and cytoplasmic fractionations were monitored, respectively, using the antibody p300(N-15) against p300 human nuclear CREB co-activators (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-tubulin (Boehringer, Meylan, France). The absence of nuclear fraction leakage into the cytoplasmic fraction was further confirmed using an immunoblot for the BRCA1 protein with anti-BRCA1 antibody OP92 (Calbiochem, Meudon, France).

Generation of antibodies for menin detection

Polyclonal anti-menin antibodies were raised in rabbits against 2 synthesized peptides, composed of 14 and 23 amino acid peptides coupled with KHL, positioned at amino acids 388–401 for C15G and 588–610 for M23C, respectively. Both anti-sera were affinity-purified with the peptides used for immunization, then tested for menin detection. Primary antibodies were diluted at 1:1,000 for C15G, 1:5,000 for M23C, 1:5,000 for unpurified pre-immune sera and 0.1 μg/ml for anti-VSV-G.

Western blot analysis

SDS-PAGE was performed as described (Sambrook *et al.*, 1989). Proteins transferred to PVDF membranes were stained against a 1% Ponceau S. solution (Sigma-Aldrich), to check loadings, and incubated with primary antibodies overnight at 4°C in a solution containing PBS, 0.1% Tween 20 and 4% milk. After washing with PBS plus 0.1% Tween 20 and incubation with conjugated secondary antibody, filters were incubated with enhanced chemiluminescent (ECL) substrate (Amersham, Les Ulis, France) and exposed to Hyperfilm (Amersham). For quantitative analysis of immunoblots using M23C, the chemiluminescence was captured and evaluated by an image analyzer (Image Reader LAS-1000; Fuji, Tokyo, Japan).

RESULTS

Generation and characterization of anti-menin antibodies

To analyze endogenous menin in cultured cells and human tissues, we have generated polyclonal antibodies directed against synthetic peptides, located at amino acids 388–401 for antibody C15G and at amino acids 588–610 for M23C. In Western blot analysis, both C15G and M23C recognized the menin fusion protein produced from the pQE-FM1 construct, while the control pQE-FM4 reacted negatively (Fig. 1*a*). A band of a similar size was also detected by the 2 antibodies in protein extracts from cell lines (Fig. 1*b*). The size of the endogenously expressed menin was further compared with that of the tagged menin expressed in Bosc cells transfected by pCI-NtM1-S. A doublet of 68 to 70 kDa was observed in Bosc cells transfected with the construct, whereas only a single band at 68 kDa was detected in non-transfected cells and cells transfected with vector only (Fig. 1*b*). The upper band was also detected, with an antibody directed against the VSV-G epitope used as a tag (Fig. 1*c*). Extracts from testis tissue and non-transfected Bosc cells gave the same signal at 68 kDa (Fig. 1*d*).

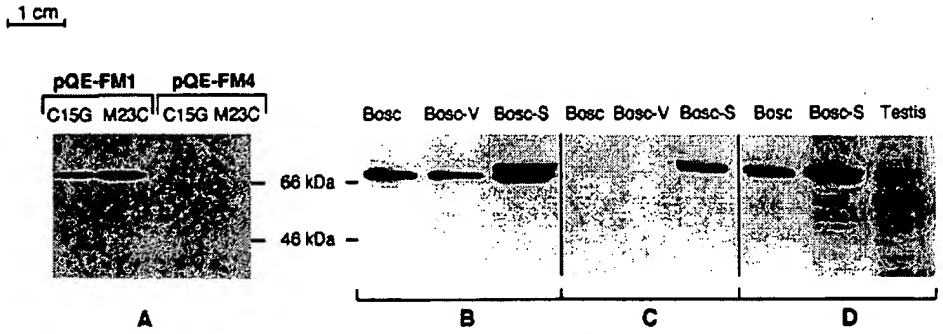


FIGURE 1 – Characterization of anti-menin antibodies. Authentication of anti-menin antibodies using a menin fusion protein *(a)*. The anti-menin antibodies used in our study, C15G and M23C, were reacted against a menin fusion protein, pQE–FM1, as well as negative control, pQE–FM4. Comparison of the gel-migration profile between endogenously expressed menin and exogenously tagged menin by immunoblot *(b–d)*. Protein (20 μg) was extracted from Bosc cells without or with vector only (Bosc-V); vectors containing tagged MEN1 cDNA in the sense direction (Bosc-S) were loaded and separated on a 10% SDS-PAGE gel. Immunoblots were first reacted with C15G *(b)* and, after stripping, with anti-VSV-G antibody *(c)*. The same amount of protein extracts from Bosc cells with or without vector containing tagged MEN1 cDNA in the sense direction and 30 μg of extracts from testis were tested using the above conditions with C15G *(d)*.

Detection of endogenously expressed menin in a panel of normal human tissues and in different cell lines

A panel of 12 normal human tissues from aborted fetuses at about 20 weeks of gestation was used for the detection of endogenous menin expression. A strong, 68 kDa, band was apparent in brain, pituitary, testis, thymus and kidney, while in thyroid only a weak band was detected (Fig. 2*a*). Several bands of other sizes, including a 40 kDa polypeptide seen in adrenal, heart and testis and a band of about 100 kDa in adrenal, were detected with both C15G and M23C; none of these bands was visualized with pre-immune sera (Fig. 2*b*). No bands were visible in liver, lung, pancreas and skin, even when 30 μg of protein extract from pancreas and skin were applied (Fig. 2*c*).

The cell lines selected for menin detection by Western blot using C15G and M23C antibodies were Bosc 23, Cos-M6, GDH, HeLa, PC12, U87 and 2 LCLs (LCL-FN1 and LCL-FN2) established from the non-affected individuals of a MEN1 family. Both C15G and M23C gave similar results. As shown in Figure 2*d*, one major band at 68 kDa was detected in all cell lines tested. An additional band at 40 kDa was visible in HeLa and U87 cells. None of the above bands was detected with pre-immune sera (data not shown).

Menin expression during the cell cycle

We investigated whether expression of menin was subject to any alteration during the cell cycle. For this purpose, HeLa cells were synchronized; harvested at time points corresponding to G_0/G_1, G_1/S, S, S/G_2 and M phases; and subsequently fractionated into cytoplasmic and nuclear extracts. Menin was apparently identical with the band detected on whole-cell extracts (Fig. 1*b*) in both nucleus and cytoplasm, though the cytoplasmic signal was weaker (Fig. 3*a*). As analyzed by autoradiography (Fig. 3*a*) and by image analysis of the chemiluminescence (data not shown), the level of menin remained approximately constant and without size alteration throughout the different cell cycle stages. A similar expression profile was obtained using synchronized NIH 3T3 cells (data not shown). The synchronization of HeLa cells was confirmed by FACS analysis (Fig. 3*c*). As a control for cell synchronization and subcellular fractionation, protein extracts were tested on immunoblot using the anti-BRCA1 antibody OP92. The variation of BRCA1 protein expression in the nucleus during cell-cycle progression (Fig. 3*b*) was as described previously (Chen *et al.*, 1996; Vaughn *et al.*, 1996).

Menin expression in LCLs carrying germ-line mutations

Two LCLs, carrying R415X and R527X germ-line mutations, respectively, were compared with 2 LCLs from non-affected individuals from a MEN1 family. Protein produced from the mutated alleles would lack both NLSs in the R415X and NLS2 in the R527X mutation. A strong signal at 68 kDa was observed in nuclear extracts of all 4 lines tested and a clearly weaker one in the cytoplasmic fractions (Fig. 4). No truncated peptide was detected with either C15G (data not shown) or M23C antibody. Expression of full-length menin was quantified as above and appeared at the same level compared with LCLs from non-affected individuals (Fig. 4).

DISCUSSION

Endogenous menin was detected in all human cell lines tested, whereas its expression appears to be more restricted in tissues. Our data are consistent with the results of transcriptional analysis using *in situ* RNA hybridization of the mouse embryos, in which a restricted transcription pattern was demonstrated at late gestational

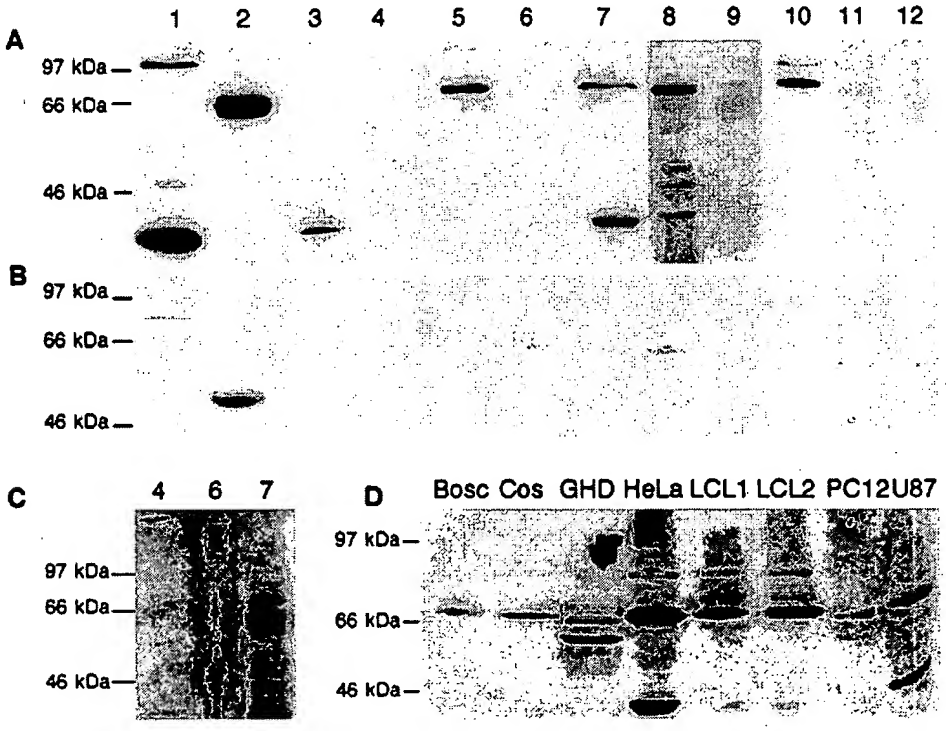
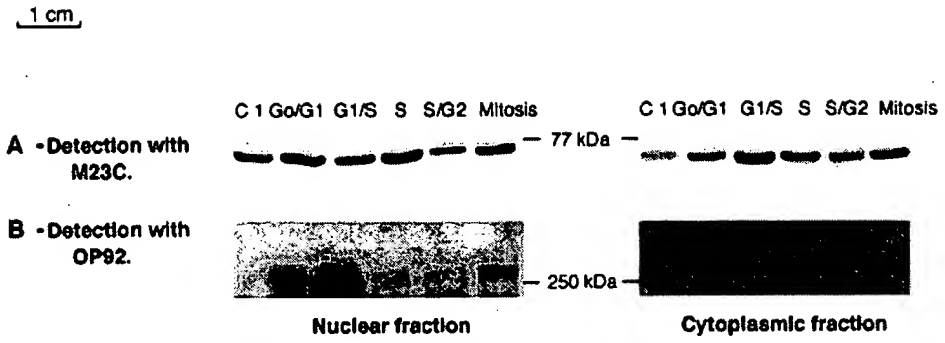


FIGURE 2 – Detection of endogenously expressed menin in normal human embryonic tissues and different cell lines by immunoblotting. For endogenous menin detection, 20 μg of protein from each of 12 human embryonic tissues at 20 weeks were used, including (1) adrenal, (2) brain cortex, (3) heart, (4) pancreas, (5) pituitary, (6) skin, (7) testis, (8) thymus, (9) thyroid, (10) kidney, (11) lung and (12) liver *(a,b)*. From pancreas and skin, 30 μg of protein were used to confirm the absence of menin in the above result *(c)*. Protein extracts (10 μg) from each of 8 cell lines *(d)* were also tested, including Bosc 23, Cos-M6, GHD, HeLa, LCL-FN1, LCL-FN2, PC12 and U87. Protein extracts were loaded and separated on a 10% SDS-PAGE gel for tissues or an 8% gel for cells and reacted to anti-menin serum *(a,c,d)* or to pre-immune sera *(b)*.



C -FACS Analysis

	C1	Starved (G0/G1)	T-0H (G1/S)	T-3H (S)	T-6H (S/G2)	Nocodazol (Mitosis)
G0/G1	60.2	**70.0**	**40.8**	**26.9**	20.9	31.5
S	19.2	20.3	**36.5**	**55.9**	33.1	18.4
G2/M	14.4	12.4	17.6	15.2	**42.8**	**43.6**

FIGURE 3 – Menin expression and its cellular location during cell-cycle progression. Cytoplasmic and nuclear protein extracts (10 μg) from synchronized HeLa cells at different stages were loaded on an 8% SDS-PAGE gel for menin detection by immunoblot with M23C *(a)*. An equal amount of the same extracts was loaded on a 6% SDS-PAGE gel and probed with an anti-BRCA1 serum, OP92 *(b)*. Markers used are RPN756 (Amersham), for detection with C15G and cytoplasmic fraction with OP92, and RPN800 (Amersham), for detection of nuclear fraction with OP92. C1 is protein extract control from the cells without treatment. The data obtained from FACS analysis of synchronized cells *(c)* show the percentage of distribution of cells in the various stages of the cell cycle.

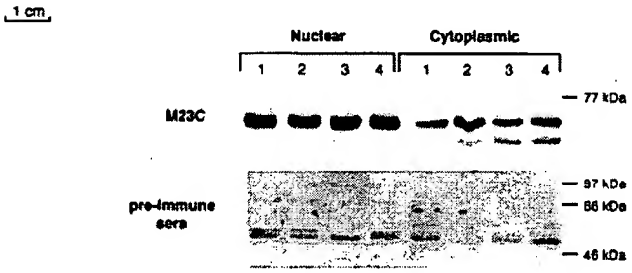


FIGURE 4 – Determination of the subcellular location of endogenous menin expressed in normal LCLs and LCLs carrying germ-line mutations removing NLS. Cytoplasmic and nuclear subcellular fractions were obtained from 2 LCLs established from the non-affected individuals of a MEN1 family (lanes 3 and 4) and 2 LCLs carrying germ-line mutations R415X (lane 2) and R527X (lane 1). Extracts (20 μg) from each line were loaded and migrated on a 10% SDS-PAGE gel. Immunoblots with the same loadings were reacted with M23C and pre-immune serum.

age (Stewart *et al.*, 1998). Expression analysis using multi-tissue Northern blots has indicated ubiquitous expression (European Consortium on MEN1, 1997), and menin protein has been detected in adult mouse pancreas (Guru *et al.*, 1999). It is possible that menin expression depends on the developmental stage, as with the *BRCA1* gene, whose expression decreases during mammary gland formation (Magdinier *et al.*, 1999). Fetal tissue samples were from gestational week 20, when the endocrine pancreas reaches the end stage of formation (Hoet *et al.*, 1995). It is also possible that the low level of endocrine cells in the fetal pancreas prevented menin from being successfully detected.

Menin is present at relatively constant levels throughout the cell cycle without undergoing any size alteration, in agreement with the data reported on NIH 3T3 cells (Guru *et al.*, 1999). This is in contrast with many proteins of the cell cycle, such as cyclins. Cell cycle–dependent post-translational modifications are, however, not excluded. Huang *et al.* (1999) have shown, by immunohistochemistry, that menin is localized mainly in the cytoplasm at M phase, as opposed to mainly in the nucleus during the other stages. We did not detect an M phase-specific increase of menin expression by Western blotting with either of our antibodies, one of which (C15G) was similar to that used by Huang *et al.* (1999). Since we used a different detection method, we do not exclude the possibility that the discrepancy is due to variation in signals other than the 68 kDa menin protein that we observed by Western blots.

We did not detect any obvious alteration in menin expression levels and cellular location in LCLs carrying germ-line mutations compared with LCLs from non-affected individuals. No truncated protein was detected in extracts from 2 LCLs carrying nonsense mutations, suggesting a fast degradation of non-functional protein. It appears that regulatory mechanisms maintain a constant level of expression, whether both alleles are functional or not. Interestingly, in sporadic parathyroid tumors, the loss of 1 allele did not result in reduced transcriptional levels (Farnebo *et al.*, 1998). Previous cytogenetic analyses have shown increased chromosomal instability in cell lines from MEN1 patients (Tomassetti *et al.*, 1995). This indicates that up-regulation of the wild-type allele may not fully compensate for loss of the other allele. The exact nature of these degradation and compensatory pathways of menin remains to be elucidated.

ACKNOWLEDGEMENTS

We thank Drs. K. O'Brien for critical revision of the manuscript; I. Callebaut and J.-P. Mornon for help on peptide selection; M. Billaud for many helpful discussions and the Bosc 23, GDH, PC12 and U87 lines; and F. Guehenneux for FACS analysis. VW was supported by a doctoral fellowship from Ligue contre le cancer de Saône-et-Loire.

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